The Role and Regulation of the Cytochrome P-450 CYP2E Subfamily

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Declaration

I declare that this thesis is my own composition and describes work carried out by myself; research carried out by, or in collaboration with, other parties is clearly acknowledged.

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ABSTRACT OF THESIS (Regulation 3.5.10)

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Several cytochrome P-450 enzymes are thought to have evolved to metabolise lipophillic xenobiotics allowing subsequent conjugatory and excretory steps to occur. The nature of the metabolic activity of these enzymes and their ability to activate a series of carcinogens has implicated them in carcinogenesis. The CYP2E subfamily activates a series of common carcinogens including N-nitrosodimethylamine (NDMA); situations therefore in which CYP2E levels are elevated may lead to increased carcinogenic risk. A mouse CYP2E cDNA and the single copy CYP2E gene were cloned and characterised and the regulation of CYP2E in this species was studied.

Mouse CYP2E protein levels were seen to be elevated by acetone in a variety of tissues; no concomitant increase in CYP2E mRNA was seen. Previous studies in the rat suggested that the CYP2E elevation resulted from substrate induced protein stabilisation. This stabilisation was suggested to be mediated by the presence of the substrate leading to the blocking of a phosphorylation event which may trigger CYP2E protein degradation. This was investigated using mutant forms of the mouse CYP2E protein lacking the phosphorylation site; in mammalian tissue culture systems both mutant and native CYP2E proteins behaved identically suggesting that the protein stabilisational event may be mediated via a mechanism other than the blocking of a phosphorylational change in the CYP2E protein.

The CYP2E mRNA and protein became elevated both in the spontaneously diabetic BB/E rat, and the mouse following starvation. As a result of increased β–oxidation in these states ketone body levels become elevated which may lead to stabilisation of the CYP2E protein. Previous studies in chemically induced diabetic rats suggested that the message elevation resulted from the stabilisation of pre-existing CYP2E transcripts; previously characterised events of this kind were seen to be mediated by elements within the 3' untranslated region (UTR) of a message. The fact that the mouse CYP2E 3' UTR is interrupted by insertion of a β2 repetitive element and that that the control transcript used in the chemically induced diabetic rat was elevated in the spontaneously diabetic BB/E rat suggested that transcript stabilisation may not be responsible for the CYP2E mRNA elevations seen in these situations. The similarity between the induction pattern of the CYP2E subfamily and several gluconeogenic enzymes, and the previous suggestion that the CYP2E subfamily may possess an endogenous gluconeogenic role, prompted the analysis of the effect of insulin on the transcriptional activity of the mouse CYP2E 5' region. The mouse CYP2E promoter was functional in a promoter-assay system but insulin had no effect on its transcriptional activity. In the BB/E rat the diabetic state also elevated the levels of several other P-450 subfamilies potentially giving an insight either into the vestigial controls operating on, or the true endogenous functions of these enzymes normally studied solely with regard to their xenobiotic metabolising role.

In order to assess the metabolic role of the CYP2E subfamily the mouse cDNA was heterologously expressed in procaryotic and eucaryotic systems and portions of the mouse gene were used in a gene-targeting experiment attempting to delete the subfamily from the mouse genome. Although unsuccessful in their original objectives, results from these studies give insights into the basis for further analysis of the role of the CYP2E subfamily.

PGS/ABST/88 *Use this side only*

Ntasesaka namb'a nkola

Ancient Mungo Proverb

(A dead elephant cannot be cut up with the finger nails)

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Abbreviations

A	Adenine
ATP	Adenosine triphoshate
bp	Base pairs
BSA	Bovine serum albumin
C	Cytosine
CAT	Cloramphenicol acetyl-transferase
C-terminal	Caboxyl-terminal
D	Daltons
δALA	δ-Amino laevulinic acid
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
ds	Double stranded
EDTA	Ethylene diamine tetraacetic acid
FCS	Foetal calf serum
G	Guanine
g	Times gravitational
GHb	Glycated haemoglobin
GST	Glutathione S-transferase
i. g.	Intragastrically
i. p.	Intraperitoneally
IPTG	Isopropyl-1-thio-β-D-galactoside
IRE	Iron responsive element
kb	Kilobase
MOPS	3-(*N*-morpholino)propane sulphonic acid
μGST	Microsomal glutathione S-transferase
M_r	Relative molecular mass
mRNA	Messenger RNA
NDMA	*N*-nitrosodimethylamine
N-terminal	Amino terminal

OD	Optical density
P-450	Cytochrome P-450
P-450 reductase	NADPH-dependent cytochrome P-450 reductase
PBS	Phosphate buffered saline
PEG	Polyethylene glyol
RNA	Ribonucleic acid
SDS	Sodium dodecyl-sulphate
SDS/PAGE	SDS polyacrylamide gel electrophoresis
SINE	Short interspersed repeat element
ss	Single stranded
T	Thymidine
TEMED	*N, N, N', N'* -tetramethylene diamine
Tris	Tris (Hydroxymethyl) amino ethane
UTR	Untranslated region
UV	Ultraviolet
v	Volume
w	Weight
X-Gal	5-bromo-4-chloro-3-indole-β-D-galactoside
+/-	With or without

Publications arising from this work

Freeman, J. E., Stirling, D. J., Russell, A. L. & Wolf, C. R. cDNA sequence, deduced amino acid sequence, predicted gene structure and chemical regulation of mouse Cyp2e1. Biochem. J. 281, 689-695 (1992).

Freeman J. E. & Wolf, C. R. Deduced amino acid sequence, predicted structural feature and proposed substrate binding residues of mouse Cyp2e1. In: Cytochrome P-450: Biochemistry and Biophysics (Archakov, A. I. & Bachmanova, G. I., eds.) pp 385-387, INCO-TNC (1992).

Table of Contents

Chapter 1: Introduction

Section 1.1: The Cytochrome P-450 multigene family

The cytochrome P-450 (P-450) multigene family encodes a series of enzymes with monomeric M_r of around 50,000 containing non-covalently bound haem. The name of this superfamily derives from the characteristic peak at 450 nm generated from the difference between the absorption spectra due to the presence of a protohaem group, produced by the enzymes when reduced and bound by carbon monoxide (Omura & Sato, 1962). Members of this multigene family are widely dispersed throughout most of the procaryotic and eucaryotic kingdoms. Although many P-450 "isoforms" (that is an individual P-450 enzyme produced by a particular P-450 gene) exist, it is thought that they are unified by their ability to catalyse a common reaction. The P-450 enzymes function as terminal electron acceptors, receiving reducing equivalents from NADPH or NADH, and their actions lead to the reductive activation of molecular oxygen. The result of the activation of oxygen leads to the insertion of one oxygen molecule into the substrate present within the active site of the P-450 enzyme. Due to the nature of this reaction, the cytochrome P-450 superfamily of enzymes have been collectively termed "mono-oxygenases" (Figure 1.1):

Figure 1.1: The basic and unifying reaction catalysed by all P-450 isoforms. RH represents the substrate acted upon by the P-450 isoform in question.

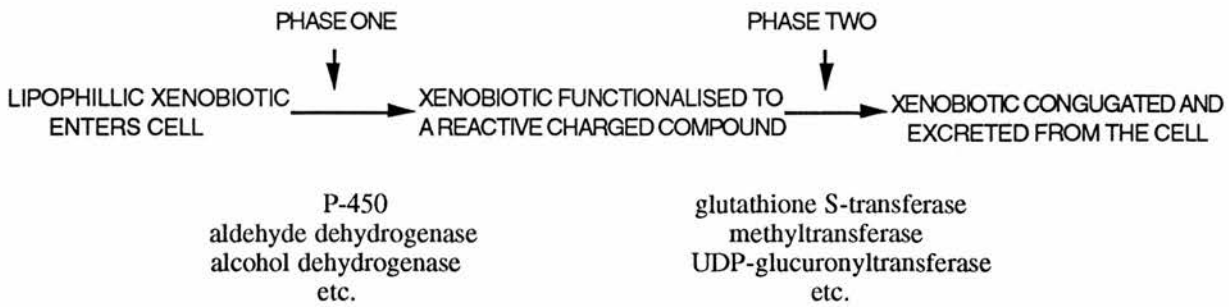
$$RH + O_2 + NADPH + H^+ \text{ -------> } ROH + H_20 + NADP^+$$

The oxygenation of the substrate can then allow a wide variety of reactions to proceed, including hydroxylation, epoxidation, per-oxygenation, deamination, desulphation, demethylation and dehalogenation (Porter & Coon, 1991). P-450 enzymes have also been demonstrated to catalyse the reductive metabolism of chemicals when the donated electrons are passed to the substrate rather than the oxygen molecule generating, in the terminology of Figure 1, $RH(H)_2$ (deGroot & Sies, 1989). In this manner the enzymes can act as oxidases, following the reduction of oxygen , generating hydrogen peroxide or water. This reaction has implicated the P-450 enzymes in oxidative stress and lipid peroxidation as a result of the radicals generated by these reactions (Gorsky *et al.*,1984).

Mammalian P-450 are found predominantly in the liver although they have also been observed to be present at lower levels in all tissues examined with the exception of striated muscle. Mammalian P-450 can be divided broadly into two classes. The first class of P-450 are those present in the mitochondrion which receive reducing equivalents from membrane bound adrenodoxin. These P-450 catalyse very specific reactions in the processes of steroid and bile acid biogenesis and their expression is localised to those tissues associated with these biosynthetic pathways. The second class of mammalian P-450 are bound to the endoplasmic reticulum and receive reducing equivalents from NADPH P-450 reductase (P-450 reductase) and in some instances cytochrome b_5. This group subdivides broadly into two types of P-450 with one group, in a similar manner to the mitochondrial P-450, catalysing very specific reactions concerned with steroid metabolism. The second group however contains P-450 isoforms capable of catalysing a diverse range of reactions involving both endogenous chemicals, drugs and xenobiotics (i.e. a foreign compound which possesses none of the capacities of the first two groups, such as environmental pollutants) as substrates. Members of this group of enzymes are often termed "xenobiotic metabolising" P-450 and each individual isoform from this group, unlike most other enzymes, has the ability to catalyse reactions involving a broad substrate range. Collectively these xenobiotic metabolising P-450 represent the most versatile biological catalysts known and the combination of all the P-450 isoforms present in an organism generates the ability to metabolise a vast array of chemicals. P-450 have been observed to be capable of catalysing the metabolism of substrates ranging in size from ethylene (M_r 28) to cyclosporin (M_r 1,201) aptly demonstrating the wide substrate range of the xenobiotic metabolising P-450 enzymes (Ryan & Levin, 1990).

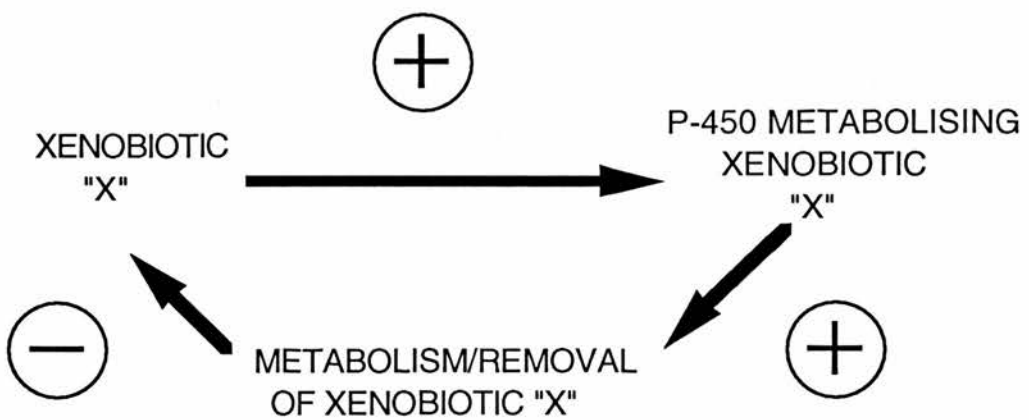
The majority of substrates metabolised by P-450 are lipophillic and one proposed role for the non-steroidigenic P-450 isoforms is in the activation of these compounds to enable their excretion; in this capacity P-450 are regarded as "Phase I" enzymes. By catalysing an oxygenation reaction on a lipophillic compound, P-450 allow the compound to become conjugated with a variety of chemical groups, such as glutathione, glucuronic acid and other sugars, methyl, acetyl and sulphate as a result of the activities of the "Phase II" enzyme systems. Enzymes of the Phase II system include the products of the glutathione S-transferase (GST), N-acetyl transferase and UDP-glucuronyl transferase gene families. The combination of these two processes allows the excretion of the resultant polar compound to occur. The Phase II conjugatory steps are spatially separate from the Phase I activation steps and the nature of the pathway(s) of conjugation which a P-450 activated, or "functionalised", compound can undergo are viewed as stochastic. (Astrom & DePierre, 1986. Gibson & Skett, 1986) (Figure 1.2).

Figure 1.2: The processes leading to the excretion of a lipophillic compound from a cell.



The activities of many of the xenobiotic metabolising P-450 are induced by the compounds which they metabolise, and this induction pattern is thought to represent part of an adaptive response which an organism can mount on exposure to xenobiotics to accelerate their excretion (Wolf, 1986) (Figure 1.3).

Figure 1.3: A feedback-loop whereby a xenobiotic may induce P-450 isoforms involved in its own metabolism thus accelerating its removal from the body:



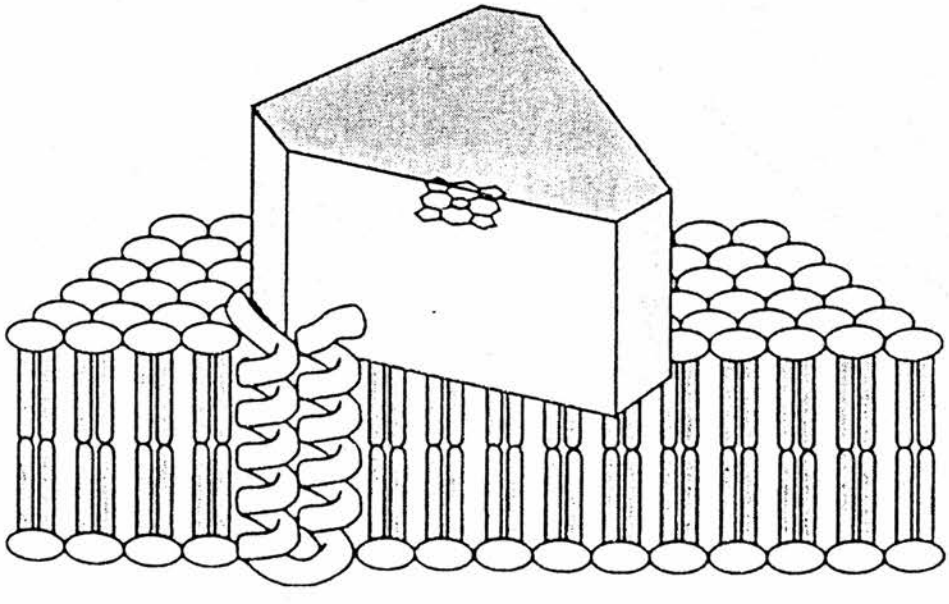
The actions of the xenobiotic metabolising P-450 can lead to both harmful and helpful consequences within an organism. A combination of the stochastic nature of Phase II conjugation reactions and the ability of many xenobiotics to induce their own metabolism can result in situations where the high energy intermediates generated by P-450 catalysed reactions are not efficiently conjugated by Phase II processes. These

reactive compounds can then react with cellular proteins, RNA and DNA and can lead to cell death, mutagenesis or the initiation of carcinogenesis. It is this potentially harmful feature of the xenobiotic-metabolising P-450 families activity which has stimulated much research into these enzymes (Wolf, 1986; Guengerich, 1988; Gonzalez, 1990; Juchau, 1990; Guengerich, 1991, Gonzalez, 1992).

Section 1.2: Cytochrome P-450 structure

A three-dimensional structure for the soluble procaryotic P-450 CYP101 (P450 cam) has been obtained from *Pseudomonas putida.* The P-450 enzyme resembles a triangular prism with an asymmetrical clustering of α-helices to one half of the molecule which also contains the haem prosthetic group (Poulos *et al.*, 1987). No direct structural data has been obtained from any of the membrane bound eucaryotic P-450. However the membrane attachment region of the eucaryotic protein is suggested to be a small membrane anchor at the N-terminus of the protein, and various studies have suggested that the rest of the protein may generate a structure equivalent to that seen in CYP101 (Nelson & Strobel, 1988; Zvelebil *et al.*, 1991). Given that all the P-450 enzymes catalyse a reaction with a common theme the suggestion that they will retain a generally similar structure to accommodate this reaction does not appear unreasonable. A diagrammatic representation of the structure of membrane bound P-450 is illustrated in Figure 1.4.

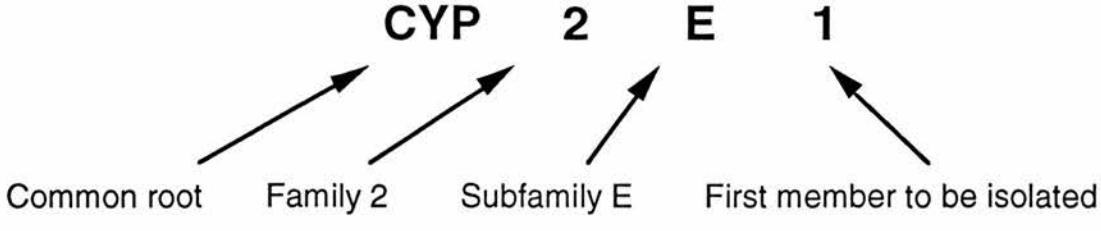
Figure 1.4: A diagramatic representation of the proposed structure and membrane attachment mechanism of membrane bound cytochrome P-450. Taken from Nelson & Strobel (1988).



Section 1.3: Cytochrome P-450 nomenclature

To aid in the distinction of particular P-450 isoforms and allow isoforms from different species to be logically related, a nomenclature system for the supergene family has been devised. The predicted or determined amino acid sequence for a new P-450 isoform is aligned with the amino acid sequences of previously determined P-450. If the new P-450 sequence shares greater than 40% similarity with any other P-450 sequence or group of P-450 sequences, it is placed in a common group with them. Collectively P-450 within a common group of this nature are described as being in the same "family" denoted by the root "CYP" and distinguished from other families by an arabic numeral. If sequences within a family share greater than 59% similarity with other members of that family, then they are placed in a subgroup of the family termed a "subfamily"; separate subfamilies are denoted by capital letters following the family numeral. Isoforms within a subfamily are assigned an additional arabic numeral according to the chronological order of their discovery. P-450 isoforms from different species, which clearly represent the species-specific version of same enzyme, are given the chronological discovery numeral of the first isoform in the subfamily to be isolated, regardless of species of origin or order of discovery, of subsequent enzymes (Figure 1.4).

Figure 1.4: An example of the application of the P-450 nomenclature system for the CYP2E1 isoform.



The mouse P-450 are distinguished from those of all other species through the use of smaller letters in both the root and subfamily delineation, for example CYP2E represents the CYP2E subfamily in all species apart from the mouse and Cyp2e represents the homologous P-450 subfamily in the mouse.

The nomenclature also distinguishes between an isoform's gene and transcript by italicising the nomenclature, for example *CYP2E1* represents either the transcript or the gene of the CYP2E1 subfamily, whereas the cDNA and protein form of the subfamily remain non-italicised.

Although the application of this nomenclature system has enabled the unification of much of the research on this superfamily, several drawbacks and anomalies are still present. The chronological assignment of subfamily members, if clear cross species homologues cannot be assigned, means that genes from different species are mixed together. The use of small letters to denote P-450 in the mouse and the italicised or non-italicised distinction of the nature of the P-450 in question can both be confusing.
This nomenclature system is used where possible throughout this work and the trivial names assigned to particular P-450 isoforms by the original investigators cited are replaced in this manner. The nature of the P-450 referred to (i.e. whether it is a gene, mRNA, cDNA or protein) is however stated rather than presented in an italicised or non-italicised form to avoid confusion. Although when a mouse P-450 is discussed in isolation small letters are used, if a subfamily is being discussed in general capital letters are employed, so for example the "CYP2E subfamily" refers to all members of that subfamily in all species including the mouse.

Section 1.4: Evolution of the P-450 superfamily

P-450 genes are widely spread throughout the procaryotic and eucaryotic kingdoms and may have originated from a single ancestral gene. Through the analysis of P-450 amino acid sequences obtained from a variety of organisms, in conjunction with information about predicted speciation times, an evolutionary mechanism leading to the generation of the P-450 superfamily has been postulated (Nelson & Strobel, 1987; Borlakoglu & John, 1989; Ronis *et al.*,1989; Gonzalez & Nebert, 1990). The enzyme encoded by the ancestral P-450 gene may have been involved in steroid metabolism and the maintainance of lipid membranes, and is thought to have undergone a duplication event approximately 1.4 billion years ago giving rise to the two classes of mitochondrial and endoplasmic reticulum attached P-450. Approximately 900 million years ago, it is predicted that several gene duplication events in the endoplasmically attached isoform gene formed the basis for the development of the steroid biosynthetic and xenobiotic metabolising P-450 forms. This latter lineage underwent a great deal of further duplication and expansion approximately 400 to 600 million years ago. This period of marked expansion of the xenobiotic metabolising P-450 is thought to have occurred in the Devonian period of the Paleozoic era and may therefore have coincided with the emergence of vertebrates onto land. The driving force behind this period of xenobiotic P-450 gene expansion was suggested to be the result of "animal-plant chemical warfare" experienced by the newly emerged land vertebrates (Gonzalez & Nebert, 1990). The expansion of xenobiotic metabolising P-450 genes may therefore have reflected the need of land vertebrates to metabolise and excrete plant xenobiotics, or

"phytoallexins"; the plants had colonised the land several million years before the emergence of the vertebrates. The expansion of the xenobiotic metabolising P-450 is thought to have arisen by gene duplication and following speciation, species unique gene duplication and gene conversion events generated species specific P-450 gene profiles. The P-450 profile present in a particular species may reflect the effect of the specific diet of that species, which then drives the expansion of P-450 families or subfamilies with metabolic activities pertinent to the xenobiotics encountered (Gonzalez & Nebert, 1990). Examples of species specific subfamily gene expansion have been seen, in the rat CYP2D subfamilies, which is suggested to have arisen by several clear duplication and conversion events leading to expansion of the CYP2D subfamily to contain at least 5 genes (Matsunaga *et al.*, 1990).

Section 1.5: Mammalian P-450 families

The nomenclature system applied to delineate the P-450 superfamily has resulted in the establishment of 10 mammalian P-450 families. These families can be broadly divided according to their function and both the specificity and nature of the reactions which they catalyse. Mammalian P-450 families 1 to 3, with the possible inclusion of family 4, are involved in the metabolise of xenobiotics and individual enzymes from these families are capable of metabolising a wide substrate range. The other 6 families (families 7, 11, 17, 19, 21 and 27) are involved in the metabolism of steroids and bile acids and catalyse very specific substrates and reactions (Table 1.1).

Table 1.1: Mammalian cytochrome P-450 families and their general metabolic activities: The numbers of subfamily members represent all those known in all mammalian species for which clear species independent homologues cannot be established. Pseudogenes and allelic gene variants are not included in this table (Table modified from Gonzalez, 1992).

P450 family	No. of subfamilies	No. of isoforms	General reactions catalysed
CYP1	1	2	Xenobiotic metabolism
CYP2	8	57	Xenobiotic and steroid metabolism
CYP3	1	10	Xenobiotic and steroid metabolism
CYP4	2	10	Fatty acid ω-and ω-1 hydroxylation
CYP7	1	1	Cholesterol 7α-hydroxylase
CYP11	2	3	Steroid 11β-hydroxylase
CYP17	1	1	Steroid 17α-hydroxylase
CYP19	1	1	Steroid to oestrogen aromatase
CYP21	1	1	Steroid 21-hydroxylase
CYP27	1	1	Cholesterol 27-hydroxylase

Section 1.5.1: Mammalian P-450 families associated with xenobiotic metabolism

The CYP1 family: The gene copy number of this family has remained evolutionarily conserved, and two enzymes, CYP1A1 and CYP1A2, are seen in all mammalian species studied. In the trout only one CYP1A gene is present, suggesting that a gene duplication event occurred after the divergence of land and sea vertebrates (Heilmann *et al.*, 1988). Induction of the CYP1A1 gene by xenobiotics has been well characterised and is seen to be mediated via a transcription factor, the Ah-receptor (Aromatic hydrocarbon), which is believed to be a member of the steroid hormone receptor superfamily (Fujisuma-Sehara *et al.*, 1988; Hoffman *et al.*, 1991).This receptor binds chemicals such as 3-methylcholanthrene and 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD), and activates transcription from the CYP1A1 gene on binding xenobiotic DNA response elements (XREs) in the promoter region of the gene. These XREs sequences are related to the glucocorticoid response elements (GREs) (Hapgood *et al.*, 1989). The CYP1A1 protein metabolises a series of xenobiotics including polyaromatic hydrocarbons such as benzo[a]pyrene. The CYP1A2 gene is also inducible by xenobiotics but this event may be mediated post-transcriptionally. CYP1A2 has been shown to metabolise several mutagens such as the arylamine 2-acetyl aminofluorene, aflatoxin B_1 and caffeine (Silver & Krauter, 1990).

The CYP2 family: This family has undergone a great deal of expansion and is now represented by 8 subfamilies. The members of this family are suggested to be principally responsible for the detoxification of xenobiotics and their expansion is suggested to reflect this role (Nelson & Strobel, 1987; Henderson & Wolf, 1992).

The CYP2A subfamily: The members of this family are associated with the metabolism of steroids with, for example, rat CYP2A1 and CYP2A2 catalysing the 7α and 15α hydroxylation of several steroids including testosterone and dihydroxy-testosterone. These reactions may play a role in the action of these hormones ; it was seen that the 7α–hydroxylated form of testosterone does not possess androgenic activity and can inhibit other enzymes associated with steroid metabolism (Sonderfan & Parkinson, 1988). The CYP2A subfamily also metabolises drugs, such as coumarin, and xenobiotics, for example some nitrosoamines (Yun *et al.*, 1992). To date three CYP2A members have been identified in the rat whereas mouse and human only possess two. Members of this family have been seen to be transcriptionally induced by 3-methylcholanthrene (Nagata *et al.*, 1987).

The CYP2B subfamily: Although the members of the CYP2B subfamily are generally regarded as being inducible by compounds such as phenobarbital, this subfamily demonstrates how members of the same gene subfamily can respond differently to inducing agents. In the rat CYP2B subfamily, the CYP2B 1, 2, and 8 genes are transcriptionally induced by phenobarbital whilst CYP2B3 is not (Giachelli *et al.*, 1989). The molecular mechanism behind the transcriptional induction of the CYP2B subfamily by compounds such as phenobarbital is unclear, and a nuclear receptor for the inducing agents, if one exists, appears to possess little affinity for the inducing chemicals (Okey, 1990). P-450 genes in *Bacillus megaterium* were also seen to be inducible by compounds such as phenobarbital. This observation suggests that the presence of common control elements in the regulation of P-450 genes across a wide variety of species has been evolutionarily conserved (He & Fulco, 1991). The CYP2B members have been shown to catalyse a variety of reactions including the *N*-demethylation of benzphetamine, the hydroxylation of hexobarbital and a series of steroid hydroxylations (Giachelli *et al.*, 1989).

The CYP2C subfamily: This subfamily is the largest of the family 2 P-450 with at least 9 genes present in the rat and 6 in the human. The activities of these proteins has been associated with the metabolism of both steroids and drugs such as mephenytoin. Although some members of the CYP2C subfamily have been shown to be inducible, for example the rabbit CYP2C 4 gene is induced by phenobarbital, most members are considered as being constitutively expressed (Hasset & Omiecinski, 1990). In the rat several members of the CYP2C subfamily demonstrate sexual dependence and dimorphism and the mechanisms for establishing these differences is been analysed (Mode *et al.*, 1988).

The CYP2D subfamily: This subfamily contains at least 5 genes in the rat although only 1 functional genes is present in the human (Matsunaga *et al.*, 1990). The subfamily does not appear to be inducible on exposure to xenobiotics, and is associated with the metabolism of steroids as well as several drugs such as debrisoquine and sparteine in humans (Gonzalez *et al.*, 1987; Gough *et al.*, 1990; Smith *et al.*, 1992).

The CYP2E subfamily: the CYP2E subfamily represents the most highly conserved family 2 subfamily, with only a single gene being observed in all species examined apart from the rabbit where the gene has recently duplicated (Khani *et al.*, 1988). The CYP2E proteins catalyse the metabolism of a series of small solvent molecules including ethanol, isopropanol, and acetone, drugs such as acetominophen and several carcinogenic nitrosamines such as *N*-nitrosodimethylamine (NDMA). Members of this subfamily are seen to be induced following exposure to several solvent molecules, starvation or in the diabetic state (Yang *et al.*, 1990). This subfamily is discussed in more detail below.

The CYP2F subfamily: A subfamily related to the CYP2A subfamily and only characterised to date in humans which appear to contain at least 2 CYP2F genes. CYP2F protein is seen to be expressed in the lung, where it has been shown to metabolise the lung toxin skatole (3-methylindole) (Thornton-Mannering *et al.*,1991).

The CYP2G subfamily: A subfamily characterised to date only in the rat where CYP2G protein has been seen to be expressed only in the nasal mucosa. This observation has led to the suggestion that this subfamily may be involved in the biotransformation and detection of odorants and olfactory sensing (Nef *et al.*, 1990).

The CYP2H subfamily: A subfamily characterised to date only in chickens where 2 members are present. The genes appear to be inducible by phenobarbital, but the reactions which they catalyse have not been extensively characterised (Hansen & May, 1989).

The CYP3 family: Members of the CYP3A subfamily are inducible by glucocorticoids and synthetic steroids such as dexamethasone and pregnenelone 16 α-carbonitrile. The human possesses at least 4 CYP3 genes and the transcripts from this family are the most abundant in the liver (Aoyama *et al.*, 1989). The CYP3A subfamily members are involved in the metabolism of steroids and it is still a matter of debate as to whether the reactions which they catalyse are purely degradative, or generate products with an intrinsic biological role. Members of the CYP3A subfamily have also been shown to metabolise drugs such as nifenipine and ethylmorphine, and xenobiotics such as aflatoxin B_1 and Cyclosporin A (Grogan *et al.*, 1990).

The CYP4 family: The CYP4 family is predicted to be very ancient and possesses 2 subfamilies which have themselves undergone some expansion in gene copy numbers. The CYP4A subfamily catalyses the ω, and ω-1 hydroxylation of short to medium chain fatty-acids such as palmitate, laurate, and arachidonate; it is not clear if this subfamily is involved in the metabolism of xenobiotics. Several members of the CYP4A subfamily are induced on exposure to a variety of hypolipidaemic drugs such as clofibrate (Hardwick *et al.*, 1986). This induction is mediated via the peroxisome proliferation activated receptor (PPAR), a member of the steroid hormone superfamily, and may form part of a gene locus associated with elevated peroxisomal activity (Reddy *et al.*, 1992; Isseman & Green, 1990). In the rat the second CYP4 subfamily, CYP4B, has been shown to metabolise xenobiotics such as aflatoxin B_1 and 2-acetylaminofluorene (Nhanburo *et al.*, 1989).

Section 1.5.2: Mammalian P-450 families associated with steroid anabolism

The endoplasmically attached CYP7, 17, 19, 21 and 27 families and the mitochondrial CYP11 family are involved in the synthesis of steroid, sex-steroid, mineralocorticoid and glucocorticoid hormones. Unlike families 1 to 4 these families catalyse very specific reactions on very specific substrates and are seen to be expressed only in tissues involved in steroid anabolism. These P-450 are generally thought not to participate in the metabolism of xenobiotics, but this view has been questioned (Niranjan *et al.*, 1988. Hollis, 1990). Unlike the xenobiotic metabolising P-450, the gene copy numbers of these gene families have not undergone significant expansion

(Al-Othman *et al.*, 1988; Fevold *et al.*,1989). Genes from the CYP 11, 17 and 21 families have been shown to be transcriptionally induced by elevations in cAMP levels on stimulation of cells with adrenocorticotrophin hormone (ACTH) (Waterman *et al.*, 1988). Several genes from these families have been sequenced, and although they were not seen to possess canonical cAMP response elements (CREs), they have been suggested to contain several novel CREs (Inou *et al.*, 1988).

Section 1.6: The role for P-450 enzymes in chemical carcinogenesis

P-450 catalysed activation of lipophillic compounds can lead to damage of cellular macromolecules and the potential initiation of carcinogenesis. Since the observation that these enzyme systems were responsible for the biotransformation and activation of carcinogenic azo-dyes (Meuller & Miller , 1948; Meuller & Miller, 1953), attempts to understand the roles which both individual P-450 isoforms, and the P-450 superfamily as a whole, play in the generation of genotoxic damage, has been a central theme to a large amount of P-450 research. Understanding physiological and environmental situations which may perturb P-450 activity levels, with the resultant modifications in potential carcinogenic risks, is also therefore of central importance in understanding their role in carcinogenesis. Research establishing roles for P-450 in the initiation of carcinogenesis has exploited both whole animal and reductionist systems, and employed both biochemical and genetic approaches.

The biochemical analysis of P-450 initially addresses the possibility that the metabolism of a compound is catalysed by P-450 and allows the nature of the products of such a reaction to be analysed. Biochemical assays have employed endoplasmic reticulum enriched fractions ("microsomes") and the involvement of P-450 in the metabolism of a chemical has been established through the use of general P-450 inhibitors, such as metyrapone and SKF525A. The potential contribution of an individual P-450 isoform in the metabolism of a chemical can then be assessed either by employing microsomes in which that particular isoform has been induced (Kaminsky *et al.*, 1983), by adding inhibitory antibodies to a specific isoform to the microsomal mixture (Edwards *et al.*, 1991), or by adding chemical inhibitors to a particular isoform (Hall *et al.*, 1987). This approach has the advantage that it does not pre-suppose which P-450 isoform may be involved in the metabolism of a chemical, and has been used to delineate the P-450 isoforms involved in the metabolism of a variety of xenobiotics for example aflatoxin B_1 (Shimada & Guengerich, 1989; Forrester *et al.*, 1990).

Alternatively, the potential reactions catalysed by an individual P-450 isoform can be studied in isolation from the rest of the P-450 population; theoretically if a panel of P-450 were generated in this manner the potential metabolism of a chemical by each

individual P-450 isoform present in the total organism could be assessed. The P-450 isoform can either be purified directly from the animal tissue in which it is endogenously expressed (Wood *et al.*, 1976; Levin *et al.*, 1986), or generated heterologously. This second heterologous expression approach has now been applied in bacterial (Barnes *et al.*, 1991; Larson *et al.*, 1991), yeast (Guengerich *et al.*, 1991b) and *Drosophila* (Jowett *et al.*, 1991) , systems and both in insect (Asseffa *et al.*, 1989; Thornton-Mannering *et al.*, 1991) and mammalian tissue culture (Zuber *et al.*, 1986, Simula *et al.*, 1993). In using this approach to determine the metabolic roles of P-450 isoforms in the metabolism of a certain chemical, it must always be remembered that, particularly in the generation of the P-450 isoform by heterologous expression, the possible use of an allelic variant of a P-450 isoform may greatly influence the results obtained.

Whole animal models have been employed in the establishment of roles for P-450 isoforms in carcinogenesis. Mouse strains were discovered which, due to presumed genetic differences in the Ah receptor protein, showed either a high, "responsive", or low, "non-responsive", induction level of CYP1A1 following exposure to a variety of polyaromatic hydrocarbons such as 3-methylcholanthrene (Nebert & Negishi, 1984). On exposure to carcinogenic polyaromatic hydrocarbons, such as benzo[a]pyrene, which is both carcinogenic and an inducer of the CYP1A1 isoform, the effect of the level of CYP1A1 induction on chemical carcinogenesis was studied. It was seen that the responsive strains, with high CYP1A1 induction, were more susceptible to fibrosarcomas and lung tumours following subcutaneous injection and tracheal instillation of the carcinogen respectively, than the non-responsive strains with lower CYP1A1 levels. Conversely it was seen that the non-responsive strains were more susceptible than the responsive strains to lymphosarcomas and leukemias as a result of subcutaneous administration of the carcinogen (Nebert & Negishi, 1984). These results are suggested to reflect the nature of the carcinogen administration. In the responsive strains, the clearance and detoxification of the carcinogen by CYP1A1 and the conjugatory systems within the liver, the "first pass" clearance, is greater than in the non-responsive strains and reduces the delivery of the carcinogen to remote sites of the body, leading to a reduced incidence of lymphomas and leukemias. Tumour generation at the site of administration in the responsive strains was higher however, due to the induction of CYP1A1 and the generation of activated metabolites at these sites in higher levels than generated at the site of administration in the non-responsive strains (Okey *et al.*, 1988).

It was observed that the the human CYP1A1 gene in primary cultured lymphocytes demonstrated a trimodal distribution of induction response with "high", "low", and "intermediate" induction levels being seen (Kellerman *et al.*, 1973). Studies suggested a

correlation between the inducibility of the CYP1A1 subfamily and the level of cancers resulting from exposure to carcinogens, such as benzo[a]pyrene, present in cigarette smoke. Smokers of high and intermediate inducible CYP1A1 phenotype displaying a 36 fold and 16 fold higher incidence of bronchial carcinoma than those with a low inducibility phenotype (Kouri *et al.*, 1982).

By studying genetic polymorphisms in P-450 genes which result in a changed metabolic activity and establishing a correlation between the modified metabolic activity and the incidence of cancer, links between P-450 isoforms and carcinogenesis can be established. Such studies provide a powerful genetic and epidemiological approach to establish links between particular P-450 isoforms and particular cancer incidence (Wolf, 1990; Smith *et al.*, 1992). An example of the use of this approach was used in the analysis of the human CYP2D locus; mutations in this locus lead to the loss of functional CYP2D protein which is reflected in an individuals reduced ability to metabolise the drugs debrisoquine and sparteine. Genotype analysis of polymorphisms within the CYP2D locus lead to establishment of a correlation between the loss of functional CYP2D subfamily activity and reduced susceptibility to smoking-induced cancers (Gough *et al.*, 1990; Smith *et al.*, 1992).

By a combination of biochemical and genetic studies, it is clear that P-450 are capable of metabolising and activating a variety of carcinogens, and it is seen that variations in the P-450 isoforms present in the total P-450 population of an organism can effect the degree of carcinogenesis experienced on exposure to foreign compounds.

Section 1.7: The CYP2E subfamily

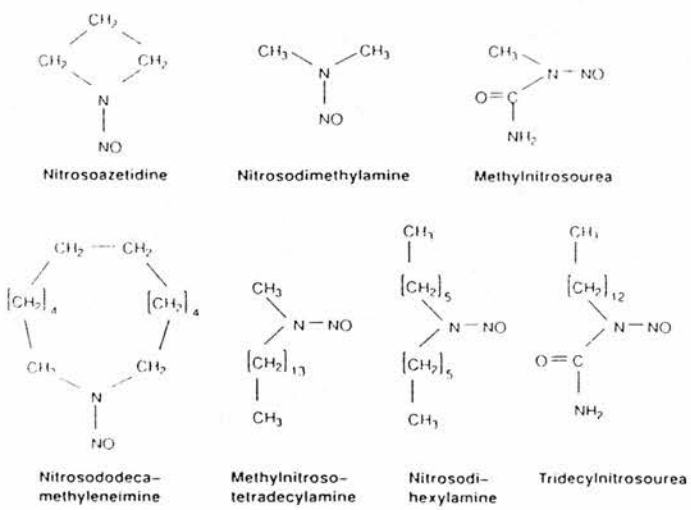
Unlike other CYP2 subfamilies the CYP2E subfamily is unique in that it is present as a single gene in most species studied; the proteins encoded by the CYP2E genes of a variety of species are highly conserved suggesting that they may play a highly conserved role in a variety of organisms. The rabbit provides an exception to this observation; in this species two genes are present which are predicted to be the result of a gene duplication event which took place around 11 million years ago, well after the predicted rabbit speciation point. The two rabbit genes however are still highly similar with only 18 amino changes present throughout the length of the 493 amino acid sequence (Khani *et al.*, 1988).

Interest in the CYP2E subfamily has mainly centred around its role in the metabolism of a series of common environmental nitrosamines which are carcinogenic, and an understanding of the control of this subfamily provides an insight into situations which may lead to an increased carcinogenic risk following exposure to these compounds.

Section 1.7.1: The role of the CYP2E subfamily in the metabolism of nitrosamines and other solvents

Several nitrosamines are metabolised by the CYP2E subfamily and this class of compounds represents one of the most potent group of carcinogens known. The carcinogenic properties of nitrosamines have been recognised for many years (Magee & Barnes, 1956), and nitrosamines have been shown in animal studies to be capable of generating every class of human cancer (Lijinsky, 1992). The chemical structures of several carcinogenic nitrosamines are illustrated in Figure 1.6:

Figure 1.6: The chemical structure of several carcinogenic nitrosamines (Taken from Lijinsky, 1992).



The majority of nitrosamines are solvents and most unsubstituted forms are liquid at room temperature whether symmetrical or unsymmetrical, cyclic or acyclic. Although some examples exist of nitrosamines being present in a native from in the environment, such as the methylnitrosourea derivative streptozotocin which is produced by the soil fungus *Streptomyces achromogenes*, most nitrosamines are formed endogenously within the body. Nitrosamines are generated following reactions between nitrites and either secondary or tertiary amines. Nitrites are used to produce cured meat and fish, and are present in beverages produced from heated malt such as beer and whisky. Nitrites are also produced within the body following the reduction of nitrates present in the environment and the diet. Amines are naturally present in foods, and are used as food additives and in agricultural, industrial and medicinal chemicals. The acidic nature of the stomach provides a perfect environment for the formation of nitrosamines from ingested amines and nitrite. Nitrosamines are also present in cured tobacco and, when either inhaled into the lungs as smoke, the nose as snuff or chewed, represent the most significant carcinogens present in these products. Occupational exposure to

nitrosamines arises in the leather tanning and rubber manufacturing industries, as well as from the general use of amines as bases in industrial synthesis and their inclusion in cutting and lubricating oils (Winn, 1984; Bartsch & Montesano; 1984. Lijinsky, 1992).

Nitrosamines need to be activated to produce their carcinogenic action through reactions leading to the generation of a reactive alkyldiazonium species capable of alkylating DNA. The nature of the alkyldiazonium species generated is dependent on the parent molecule so, for example, the activation of *N*-nitrosodimethylamine (NDMA) generates methylated purine and pyrimidine nucleotides upon activation, whereas *N*-nitosodiethylamine (NDEA) generates ethylation products (Lijinski, 1992). Given the widespread distribution of these compounds in the environment and the ease of their endogenous formation, coupled with their potent carcinogenic actions, the enzymes capable of catalysing these activation reactions are of extreme importance.

Studies on the activation of NDMA by demethylation implicated P-450 enzymes in catalysing this reaction. It appeared that several enzymes were involved in the demethylation of NDMA as multiple K_m values for this reaction were obtained (Czygan *et al.*, 1973). The enzyme with the lowest K_m in the demethylation of NDMA, approximately 20 μM, was purified from rat liver and was shown to be CYP2E1 (Patten *et al.*, 1986). The other enzymes with higher K_m values (0.3-0.5 mM) for the demethylation of NDMA were later seen to be members of the CYP2A subfamily (Yun *et al.*, 1992).

The rat CYP2E1 enzyme was shown to be capable of activating a series of other nitrosamines including NDEA and *N*-nitrosobutylmethylamine (NBMA) and it appears that the steric constraint engendered by the alkyl groups determine the ability of the nitrosamine to enter the CYP2E1 active site and undergo activation (Lee *et al.*, 1989).

As well as several nitrosamines, the CYP2E subfamily metabolises a series of small solvents, for example, alcohols (Morgan *et al.*, 1982), isopropanol, acetone (Koop & Cassaza, 1986), benzene (Johansson & Ingelman-Sundberg, 1988), *p*-nitrophenol (Koop, 1989), aniline (Patten *et al.*, 1986), carbon tetrachloride (Johansson & Ingelman-Sundberg, 1985), chloroform and several ethers (Pantuck *et al.*, 1988. Brady *et al.*, 1988). The CYP2E subfamily has also been shown to metabolise several common drugs such as acetominophen (Koop *et al.*, 1982).

The capacity of the CYP2E subfamily to activate several carcinogenic nitrosamines as well as its role in the metabolism of hepatotoxic chemicals such as carbon tetrachloride and acetominophen, means that the study of this subfamily, and the nature of its regulation, is of potential importance in understanding the carcinogenic and other injurious actions mediated by these common xenobiotics.

Section 1.7.2: Induction of the CYP2E subfamily

In common with the observation that many xenobiotics induce the levels of the P-450 isoforms which lead to their metabolism, the CYP2E subfamily protein levels were seen to be induced by solvents such as ethanol and acetone. In trivial terminology the CYP2E subfamily was previously described as the "ethanol-inducible" P-450. Solvent exposure has been shown to generate increased levels of CYP2E subfamily protein in all species studied, including the rat (Miller & Yang, 1983), chicken (Sinclair *et al.*, 1985), rabbit (Koop *et al.*, 1982), hamster (Kubota *et al.*, 1988), timber wolf (Hogy & Crankshaw, 1992), and human (Perrot *et al.*, 1989). Induction by solvent exposure was noted in a variety of tissues other than the liver, including the rabbit kidney (Ueng *et al.*, 1987) and bone-marrow (Schier *et al.*, 1989). Protein from the CYP2E subfamily has been immunohistochemically detected in the rat brain; the observed localisation of the CYP2E protein to the basal ganglia, frontal cortex and hippocampus is intriguing as it is these regions which display biochemical and morphological changes following alcohol consumption in experimental conditions (Hansson *et al.*, 1990).

No concomitant increase in CYP2E mRNA levels are observed on exposure to CYP2E protein-inducing solvents; it is thought that the induction seen relates to substrate-induced stabilisation of pre-existing CYP2E protein (Song *et al.*, 1989). It has been suggested that the substrate may induce increased stability of the CYP2E protein by blocking a phosphorylation event at a conserved Serine residue; phosphorylation at this site is suggested to lead to degradation of the CYP2E protein by a rapid proteolytic system (Ingelman-Sundberg *et al.*, 1992). There is also evidence that solvent induction may lead to an increased translation rate of the CYP2E subfamily mRNA and a fall in mRNA levels following induction (Kim & Novak, 1990; Kim *et al.*, 1990).

The CYP2E subfamily has been seen to be induced by starvation and in the diabetic state in both the rat (Young & Yang, 1983; Song *et al.*, 1987) and the human (Song *et al.*, 1990). In both these cases an elevation of both the CYP2E1 mRNA and protein was observed. It has been suggested that ketone bodies generated as a result of increased β-oxidation in both these states leads to a stabilisation of the CYP2E1 protein in a similar manner to that observed following exposure to solvents (Miller & Yang, 1986). The elevation of the mRNA in these states has been suggested to result from a stabilisation of pre-existing CYP2E1 mRNA (Song *et al.*, 1987) although this point is far from clear and a transcriptional role for the elevation of CYP2E subfamily transcripts cannot be firmly ruled out.

The CYP2E subfamily has been seen to be developmentally regulated in several species including the human (Jones *et al.*, 1992), rat (Umeno *et al.*, 1987) and rabbit (Bonfils

al., 1990) where it is seen that the gene becomes demethylated and transcriptionally active immediately after birth.
Potentially species specific controls are also seen to operate on the CYP2E subfamily; examples of this type of control are seen in the mouse kidney where a sexual dimorphism exists with higher levels being present in the male than the female (Hong *et al.*, 1989) and in the rat where the gene appears to be repressed by growth hormone (Yamazoe *et al.*, 1989a), a control not observed in the mouse for example (Henderson *et al.*, 1990).

Section 1.7.3: A role for the CYP2E subfamily in intermediary metabolism?

The observed induction of the CYP2E subfamily by solvents, starvation and diabetes led to the suggestion that the common effector generating stabilisation of the CYP2E protein in all these situations was acetone. The CYP2E subfamily was seen to catalyse the conversion of acetone to acetol and methylglyoxal, both of these products can potentially act as gluconeogenic precursors. This observation suggested that the CYP2E subfamily may participate in a gluconeogenic pathway leading ultimately to the gluconeogenic conversion of acetone, generated as a result of elevated β-oxidation in starvation and diabetes, to glucose (Koop & Cassaza, 1986). Therefore acetone and the other solvents which were observed to induce the CYP2E subfamily may exert their effect by mimicking the actions of endogenously produced ketone bodies.
This suggestion emphasises how an understanding of the potential endogenous roles of xenobiotic metabolising P-450 enzymes can lead to a better appreciation of situations in which their activities may become elevated. The observation that superficially unrelated situations, such as solvent exposure and starvation, both produce an elevation in the activity of the CYP2E subfamily can be rationalised once it is appreciated that induction in both cases may be generated by control mechanisms relating to the potential gluconeogenic role of this subfamily.

Section 1.8: Aims and Objectives

The CYP2E subfamily clearly may play a role in the carcinogenicity resulting from exposure of an organism to a variety of common environmental chemicals such as nitrosamines. Therefore understanding both the mechanisms by which this subfamily is controlled and the contribution which it makes to carcinogenesis are of clear importance.

The regulation of the CYP2E subfamily would be assessed using the spontaneously diabetic BB/E rat. The effect of the diabetic state on the CYP2E subfamily would be examined and the proposed mechanism of induction of the CYP2E subfamily in this situation reviewed. This study would be combined with the analysis of changes in other P-450 families and enzyme systems associated with xenobiotic metabolism, allowing a wider view of the effects which the metabolic status could produce on the xenobiotic metabolising capacity of an organism.

The presence of the CYP2E subfamily in the mouse would be firmly established and the cDNA and gene of the Cyp2e member in this species obtained. This analysis would establish the potential level of conservation of the CYP2E subfamily in this species and so the potential conservation of metabolic function. The regulation of the Cyp2e subfamily in the mouse would be studied both following exposure to chemicals and on starvation. The molecular basis for the regulation of the CYP2E subfamily would be studied by analysis of the flanking region of the Cyp2e gene.The potential role of phosphorylation in the control of the CYP2E subfamily protein would be assessed using Cyp2e1 proteins with mutations at the proposed controlling phosphorylation site and observing the effect which these changes produce on the accumulation of Cyp2e protein in heterologous systems.

The role of the CYP2E subfamily in carcinogenesis and the metabolism of xenobiotics would be analysed using two approaches. Firstly the metabolism of nitrosamines and other compounds would be assessed through the use of heterologous expression systems producing Cyp2e1 protein following expression of the Cyp2e1 cDNA. Secondly, using the Cyp2e gene to generate a vector targeting homologous recombination within the mouse genome, a mouse-line in which the Cyp2e gene has been removed would be generated and so the role of the CYP2E subfamily gene in this species in both intermediary metabolism and carcinogen activation could be analysed by abstraction.

The combination of these objectives will lead to a clearer understanding of the level of conservation of the CYP2E subfamily enzymes and their control between species. Such an understanding will allow a better appreciation of the situations which will lead to increased carcinogenic risk as a result of the metabolic activities catalysed by the CYP2E subfamily.

Chapter 2: Materials and Methods

Section 2.1: General comments

Chemicals and reagents were obtained from BDH and all non-commercial materials are acknowledged; all manipulations were carried out at room temperature unless otherwise stated. All media and chemicals were either purchased in a sterile condition or sterilised prior to use in an autoclave (Laboratory equipment 225 EH autoclave) or after passage through 0.2μm disposable filters (Millipore).

Section 2.2: Bacterial strains, growth media and antibiotics

Section 2.2.1: Bacterial strains used in this study

E. coli : JM109 — *rec* A1 *sup* E44 *end* A1 *hsd* R17 *gyr* A96 *rel* A1 *thi* Δ (*lac -pro* AB)
F'[*tra*D36 *pro*AB$^+$*lacI*^{q} *lacZ* ΔM15]
Used as a general host for recombinant manipulation and as a host in heterologous expression.

HB101 — *sup* E44 *hsd* S20(r $_B$- m $_B$-) *rec* A13 *ara* -14 *pro* A2 *lacY* 1 *gal K* 2 *rps* L20 *xyl*- 5 *mtl*- 1
Used as a host strain for recombinant manipulations involving the pCAT (Promega) plasmids.

BL21(DE3) — *hsd* S *gal* (λcI *ts* 857 *ind* 1 *Sam* 7 *nin* 5 *lac* UV5-T7 gene 1) pLysE
Used in heterologous expression studies.

TG1 — *sup E* 44 *hsd* Δ5 *thi* Δ (*lac-pro*AB)
F' [*tra D*36 *proAB* $^+$ *lacI*^{q} *lac Z* ΔM15]
Used in the preparation of single-stranded DNA

BW313 — *HfrKL* 16 *Po* /45 *thi* -1 *dut* -1 *ung* -1 *supE* 44
Used to generate dUTP insertion into single-stranded DNA for site-directed mutagenesis.

XL1-Blue — *sup E* 44 *hsd R* 17 *recA* 1 *endA* 1 *gyrA* 46 thi *relA* 1 *lac*$^-$
F' [*proAB*$^+$ *lacI*^{q} *lacZ* ΔM15 Tn*10* (*tet* ^{r})]
Used to plate and propagate λZAP II bacteriophage.

Y1090 — *supF hsdR ara* D139 Δ*lon* Δ*lac* U169*rpsL* *trpC* 22::Tn10 (*tet* ^{r}) pMC9
Used to plate and propagate λgt11 bacteriophage.

Q359 — *supE hsdR* Φ80^r P2
Used to plate and propagate λEMBL3 bacteriophage.

S. typhimurium : TA1538 and LR5000 strains, as described in Ames *et al.* (1979), were used as hosts for heterologous expression.

Section 2.2.2: Bacterial culture media

L-broth/litre: 10 g Bactotryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl

L-agar/litre: As for L-broth with an additional 15 g/litre agar (Difco).

Minimal agar/litre: 200 ml 5 X M9 salts (64 g $NaHPO_4.7H2O$, 15 g KH_2PO_4, 2.5 g NaCl, 5 g NH_4Cl, 20 ml 20% glucose [w/v]), supplemented with maltose to 0.2% (w/v) and $MgSO_4$ to 10 mM. This agar was used to ensure retention of the F'episome in bacterial strains.

2 X TY/litre: 16 g Bactotryptone, 10 g yeast extract, 5 g NaCl

H-top agar/litre: 10 g Bactotryptone, 8 g NaCl, 6 g agarose (Sigma).

Section 2.2.3: Antibiotics

Ampicillin: Stock of 50 mg/ml in distilled water, used at a working concentration of 50 µg/ml in both plates and media.

Chloramphenicol: Stock of 34 mg/ml in ethanol, used at a working concentration of 25 µg/ml.

Section 2.3: DNA isolation and analysis

Section 2.3.1: Plasmid vectors used in this study

Vector	Use
pUC 18 and 19 (BCL)	General cloning
pCITE-2a (Novagen)	General cloning; has a very large polylinker
pKK223-3(mod) (Pharmacia)	Bacterial expression
pET15b (Novagen)	Bacterial expression
YepGal	Yeast expression
pMA56	Yeast expression
pVT100U	Yeast expression
pYeDP1-10	Yeast expression
pCAT-Basic	Mammalian test-promoter assay
pCAT-Control	Mammalian control-promoter assay
pCMV4	Mammalian expression
pHRPNS$_{TK}$	Mammalian positive-negative selection

Section 2.3.2: Phagemid vectors used in this study

Vector	Use
pTZ18 and 19 R (Pharmacia)	General cloning and generation of ssDNA
pBS SKII(-) (Stratagene)	Generated by *in vivo* excision of λZAP II ; generates ssDNA

Section 2.3.3: Filamentous bacteriophage vectors used in this study

Vector	Use
M13mp18 and 19 (BCL)	Generates ssDNA

Section 2.3.4: Double-stranded DNA isolation from plasmid and phagemid sources

Section 2.3.4a: DNA isolation from small scale (up to 2 ml) bacterial cultures

DNA was isolated from small scale cultures using a modification of the method of Birnboin and Doly (1979). Bacteria from an overnight culture were harvested by centrifugation at 12,000 g for 5 minutes and resuspended in 100 µl of Solution 1 (25 mM Tris.HCl (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM glucose). The cells were lysed on ice by addition of 200 µl of Solution 2 (0.2 M NaOH, 1% SDS[w/v]) and subsequently 150 µl of 3 M potassium acetate (pH 4.8), and incubated for 5 minutes.

The bacterial genomic DNA and debris were removed by centrifugation at 12,000 g for 5 minutes. The plasmid DNA was precipitated from the supernatant on addition of 2 volumes of ethanol and centrifugation at 12,000 g for 5 minutes. The DNA pellet was resuspended in in 10 µl of water.

Section 2.3.4b: DNA isolation from medium (up to 150 ml) and large scale (above 150 ml) bacterial cultures

Qiagen DNA affinity columns (Qiagen inc.)were used in the isolation of plasmid DNA from medium and large scale bacterial cultures. Steps in the procedure were scaled up accordingly from the protocol described above up to the addition of 3 M potassium acetate (pH 4.8) where the sample was immediately centrifuged for 30 minutes at 20,000 g. The supernatant containing the plasmid DNA was applied to an appropriately sized Qiagen column which had been pre-equilibrated with buffer QBT (750 mM NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) ethanol, 0.15% (v/v) Triton X-100), and allowed to enter the resin under gravity. The column was washed with 3 column volumes of buffer QC (1M NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) ethanol) during which period the plasmid DNA remains attached to the resin. The DNA was eluted from the column with 1.5 column volumes of high pH buffer QF (1.25 M NaCl, 50 mM MOPS (pH 8.2), 15 % (v/v) ethanol). The plasmid DNA was precipitated from the eluant on addition of 0.7 volumes of isopropanol and collected by centrifugation at 20,000 g for 30 minutes. The DNA pellet was resuspended in 20 µl of water per 100 ml of initial culture.

Section 2.3.5: Genomic DNA isolation from mammalian tissues

Liver tissue was weighed and 10 volumes of extraction buffer (10 mM Tris.HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 20 µg/ml pancreatic RNase, 0.5% (w/v) SDS) were added and the sample mechanically homogenised using a Silverson Laboratory mixer emulsifier. The sample was incubated at 37 ^{o}C for 1 hour following proteinase K addition to a concentration of 100 µg/ml. The sample was mixed with an equal volume of phenol equilibrated with 0.5 M Tris.HCl (pH 8.0) by inversion for 10 minutes. The aqueous and phenol phases were separated by centrifugation at 5,000 g for 15 minutes. The upper aqueous layer was transferred to a separate tube using a wide-bore pipette to avoid shearing the DNA present in this fraction and the phenol extraction procedure was repeated two further times. 0.2 volumes of 10 M ammonium acetate and 2 volumes of ethanol were added to the aqueous phase and mixed to precipitate the genomic DNA. The DNA was spooled using a glass rod, washed with 70% ethanol and allowed to air

dry before being resuspended in 10 mM TE (pH 8.0) (10 mM Tris.HCl (pH 8.0), 1 mM EDTA [pH 8.0]).

Section 2.3.6: DNA and RNA concentration estimation

Spectrophotometric readings of a known dilution of a DNA or RNA sample were taken at 260 nm and 280 nm. An A_{260} reading of 1 corresponds approximately to 50 µg/ml for double stranded DNA and RNA and 40 µg/ml for single stranded DNA. An estimate of sample purity is provided by the ratio of the absobance values at 260 nm and 280 nm value, pure DNA and RNA having a value of 1.8 and 2.0 respectively.

Section 2.3.7: Restriction endonuclease analysis of DNA samples

Restriction endonucleases were obtained from Boehringer Mannheim (BCL), New England Biolabs, Bethesda Research Laboratories (BRL) or Amersham International (Amersham). Digestions were carried out using the manufacturer's recommended buffer and conditions.

Section 2.3.8: Agarose gel electrophoresis of DNA

DNA samples were analysed using horizontal gel electrophoresis tanks (BRL). Agarose gels were prepared from either normal grade or low-melting point (Ultra Pure) agarose for diagnostic and preparative gels respectively. Agarose was used at a variable concentration between 0.6 and 1.0% (w/v) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA, [pH 8.0]). 0.1 volumes of gel-loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 15% (w/v) ficoll, in water). Following electrophoresis DNA samples were stained with ethidium bromide by addition of ethidium Bromide to a concentration of 0.5 µg/ml from a 10 mg/ml stock, and visualised using a short wavelength trans-illuminator.

Section 2.3.9: Non-denaturing poly-acrylamide gel electrophoresis (PAGE) of DNA

9% (w/v) PAGE gels were prepared (8.7% acrylamide, 0.3% *N, N'*-methylene-bis-acylamide) in TBE (0.09 M Tris.Borate, 0.002 M EDTA, pH 8.0). Ammonium persulphate (1% [w/v]) and *N, N, N', N'*-tetra-methyl ethylenediamine (TEMED) were added to 0.05% (v/v). The gels were prepared in BioRad Protean 2 vertical electrophoresis equipment.

Section 2.3.10: DNA size markers

Sizes in base-pairs; * indicates the inverted doublet of the RF ΦX174/ *Hae* III markers.

1Kb ladder: (BRL)	RF ΦX174/*Hae* III (BRL)
12, 216	1, 353
11, 198	1, 070
10, 180	872
9, 162	603
8, 144	310
7, 126	271*
6, 108	281*
5, 090	234
4, 072	194
3, 054	118
2, 036	72
1, 635	
1, 018	
516	
394	
344	
298	
220	
200	
154	
142	
75	

Section 2.3.11: Southern Blotting of DNA samples

DNA samples were separated on an agarose gel, stained with ethidium bromide and photographed. Following this the gel was washed in several changes of water and was soaked for 45 minutes in denaturing solution (1.5 M NaCl, 0.5 M NaOH), and neutralising solution for 45 minutes (3 M NaCl, 0.5 M Tris. HCl, [pH 7.5]). Gels containing mammalian genomic DNA were depurinated by an initial soaking in 0.1 M HCl for 10 minutes to increase the efficiency of transfer of higher molecular weight DNA. DNA fragments were transferred to Hybond-N filters (Amersham) in 20 x SSC (3 M NaCl, 0.3 M sodium citrate) by capillary transfer according to the method of Southern (1975). The transferred DNA fragments were cross-linked to the nylon membrane by ultra-violet irradiation (254 nm) for 30 seconds using a Stratalinker 2400.

Section 2.4: Recombinant DNA techniques

Section 2.4.1: Bacteriophage λ libraries used in this study

Mouse line	Bacteriophage λ vector	*E.coli* host
BALB/c	λZAPII	XL1-Blue
C57BL/6	λgt11	Y1090
BALB/c	λEMBL3	Q359

Section 2.4.2: cDNA library synthesis

Section 2.4.2a: Total RNA isolation

0.5g pieces of male BALB/c mouse liver were placed in 2.5 ml of Solution A (6 M guanadinium hydrochloride, 5 mM sodium citrate (pH 7.0), 0.1 M β-mercaptoethanol, 0.5% Sarkosyl [v/v]) and homogenised in a Silverson Laboratory homogeniser-emulsifier. Insoluble material was removed from the homogenate by centrifugation at 5, 000 g for 5 minutes. 1g of CsCl (BCL, Molecular Biological Grade) was dissolved into each 2.5 ml aliquot of the homogenate and the sample was layered onto a 5.7 M CsCl, 0.1 M EDTA (pH 7.5) cushion in polyallomer tubes. The homogenate was centrifuged at 125,000 g at 20 °C for 21 hours in a swinging bucket rotor. The bouyant density of RNA in CsCl is much greater than that of other cellular macro-molecules and so RNA pellets during centrifugation; the genomic DNA collects as a band in the lower portion of the CsCl gradient. Following centrifugation the supernatant was aspirated and the bottom of the polyallomer tube, containing the RNA pellet , was sliced away using a scalpel. The RNA pellet was resuspended in 1 ml of TE (10 mM Tris.HCl (pH 7.4), 1 mM EDTA) by repeated pipetting. The RNA solution was heated at 65 °C for 10 minutes and kept on ice.

Section 2.4.2b: Poly $(A)^+$ RNA isolation

Oligo (dT)-cellulose spun columns (Pharmacia) were equilibrated with 1 ml of high salt buffer (10 mM Tris.HCl (pH 7.4), 1 mM EDTA, 0.5 M NaCl) which was allowed to pass into the resin under gravity. 0.2 ml of sample buffer (10 mM Tris.HCl (pH 7.4), 1 mM EDTA, 3 M NaCl) was added to each 1.0 ml of total RNA in TE. 0.25 ml of this solution was added to the equilibrated column and allowed to enter the resin under gravity. The column was centrifuged at 350 g for 2 minutes in a swinging bucket rotor

and washed and centrifuged as above following sequential addition two 0.25 ml aliquots of high salt buffer and two 0.25 ml additions of low salt buffer (10 mM Tris.HCl (pH 7.4), 1 mM EDTA, 0.1 M NaCl); the eluant from these manipulations was discarded. The poly $(A)^+$ RNA bound to the oligo (dT)-Cellulose was eluted in four sequential 0.25 ml additions and centrifugations of TE (10 mM Tris.HCl (pH 7.4), 1 mM EDTA) which had been pre-warmed at 65 °C. This entire procedure was repeated using a fresh oligo (dT)-Cellulose column and the poly$(A)^+$ RNA enriched eluant from the first column. The RNA concentration was estimated spectrophotometrically (Section 2.3.6).

Section 2.4.2c: cDNA synthesis

5 µg of poly $(A)^+$ RNA was mixed with water to produce a final volume of 20 µl, heated at 65 °C for 10 minutes and cooled on ice. 13µl of First-strand mix, a pre-made mixture of Maloney Murine Leukemia virus (MMLV) reverse transcriptase, BSA, oligo $d(T)_{12-18}$ primer, dithiothrietol and dNTPs (Pharmacia) was added to the poly $(A)^+$ RNA and incubated at 37 °C for 1 hour during which period the oligo $d(T)_{12-18}$ primer anneals to the poly-(A) tail of most mRNAs and the cDNA first-strand is synthesised by the MMLV reverse transcriptase. This sample was added to 77 µl of pre-made second strand mix, containing *E. coli* RNase H, *E. coli* DNA polymerase I and dNTPs (Pharmacia) at 12 °C for 1 hour and 22 °C for 1 hour after which 1 unit of Klenow fragment was added and the incubation continued for 30 minutes at 37 °C. During this period the RNA: DNA duplex is nicked by the RNase H creating RNA 3' termini which are used by DNA polymerase I to catalyse second DNA strand synthesis. The addition of the Klenow fragment assures that most of the resultant cDNA molecules have non-recessed, "blunt", 3' and 5' termini required for subsequent ligation steps. The sample was heated at 65 °C for 10 minutes and the protein extracted by addition of 100 µl phenol/chloroform (1: 1 [vol/vol]). The phases were separated by centrifugation at 12, 000 g for 5 minutes and the upper cDNA containing aqueous phase removed.

Section 2.4.2d: Size selection of cDNA products

As well as full-length cDNA products cDNA synthesis produces a mass of smaller partial cDNA products generated either from degraded mRNA templates or resulting from a variety of technical problems in cDNA synthesis from full-length mRNA templates. To remove these smaller partial fragments the products from the cDNA synthesis were passed through a Sephacyl-S400 spun column (Pharmacia). the column

was pre-equilibrated in ligation buffer (66 mM Tris.HCl (pH 7.6), 1 mM spermidine, 10 mM $MgCl_2$, 15 mM dithiothrietol, 0.2 mg/ml BSA) and the cDNA solution allowed to enter the resin under gravity. The larger cDNA products (above 400 base-pairs) were eluted from the column by centrifugation at 400 g for 2 minutes. Elution in ligation buffer means that the subsequent adaptor addition step can proceed directly without the need to precipitate and resuspend the cDNA molecules.

Section 2.4.2e: Addition of *Eco* R I/ *Not* I adaptors to the cDNA products

5 µg of *Eco* R I/ *Not* I adaptors and 3 units of T4 DNA ligase were added to the 100 µl eluant from the size selection Sephacyl-S400 spun column and ligated overnight at 12 °C. By ligating the cDNA molecules into an oligonucleotide pair which form an-effectively pre-digested *Eco* R I restriction endonuclese site the cDNA molecule can be ligated directly into the *Eco* R I site of a vector without the need for methylation treatment and digestion of a conventional linker.

Eco R I/ *Not* I adaptors:

A ATT CGC GGC CGC
GCG CCG GCG$_P$

The ligation mixture was heated at 65 °C to denature the ligase and the 5 ' extended terminal of the *Eco* R I/ *Not* I adaptors was phosphorylated on addition of 10 units of polynucleotide kinase and dATP to a final concentration of 1 µM; the sample was incubated at 37 °C for 30 minutes. The cDNA sample was phenol/chloroform extracted (Section 2.11.1) and added to a non-size selecting Sephacryl S-300 spun column (Pharmacia) pre-equilibrated in STE (150 mM NaCl, 10 mM Tris.HCl (pH 7.5), 1 mM EDTA).

Section 2.4.2f: Insertion of adaptor-ligated and phosphorylated cDNA molecules into the bacteriophage vector λZAP II

2 µg aliquots of *Eco*R I digested and alkaline phosphatase treated (Section 2.11.4) λZAP II bacteriophage arms (Statagene) were mixed with 30 µl, 15 µl, and 10 µl aliquots of the final cDNA in STE buffer. The DNA in these titrations was precipitated at -70 °C for 15 minutes following addition of 1 µl of 3 M Sodium acetate (pH 7.0) and 60 µl of ethanol. The DNA was collected by centrifugation at 12,000 g for 15 minutes and resuspended in 10 µl of ligation buffer (200 mM Tris.HCl (pH 7.6), 50 mM

$MgCl_2$, 50 mM dithiothrietol), 1unit of T4 DNA ligase was added and the sample ligated overnight at 12 °C.
Titrations of the cDNA: vector ligations were employed as, although the absolute DNA concentration in the cDNA mix was known, the efficiency of the adaptor addition and phosphorylation steps were not.

Section 2.4.2g: *In vitro* packaging of the cDNA/λZAP II bacteriophage arm ligation reactions

The cDNA/λZAP II bacteriophage arm ligation reaction was mixed with 15 µl aliquots of packaging protein extracts (Stratagene). These extracts are prepared from bacteria harbouring 2 bacteriophage λ mutants which are incapable of generating packaging λDNA in isolation but are complementary in combination. The samples were incubated for 2 hours at 22 °C during which time coat proteins containing the recombinant cDNA containing bacteriophage are generated.

Section 2.5: Screening and isolating recombinant bacteriophage λ clones

Section 2.5.1: *E. coli* host preparation and titration of recombinant-bacteriophage λ libraries

The *E. coli* cells relevant to the recombinant-bacteriophage vector employed were grown overnight in L-broth supplemented with 0.2% maltose and 10 mM $MgSO_4$. The cells were collected by centrifugation at 5,000 g for 5 minutes and resuspended in 10 mM $MgSO_4$ to give a final OD_{600} of 2. Addition of maltose to the media induces the maltose operon which includes the *lam B* gene encoding the λ receptor on the surface of the *E. coli*. 10 fold serial dilutions of the recombinant-bacteriophage λ stock to be titrated were made in SM (5.8 g NaCl, 2 g $MgSO_4$, 50 ml 1 M Tris.HCl (pH 7.5) /litre) to a final volume of 100 µl. These were mixed with 100 µl of plating cells and incubated at 37 °C for 10 minutes to allow the recombinant-bacteriophage to adsorb to the *E. coli*.. The mixture was mixed with 3 ml of molten H-top (47 °C) and poured onto L-agar plates, allowed to set and the plates were inverted and incubated at 37 °C overnight. The plaque forming unit (pfu/ml) concentrations of the recombinant-bacteriophage stocks were determined from the number of plaques present on the resultant *E. coli*. lawns.

Section 2.5.2: Plating and screening recombinant-bacteriophage λ libraries

Using the known pfu/ml value of the recombinant-bacteriophage stock known dilutions of bacteriophage were mixed with host *E. coli* cells to generate a near confluent plaque density on a 20 cm^2 bacterial lawn and screened in a similar manner to that described by Benton and Davis (1977). For the λEMBL3 genomic library, and the cDNA libraries, 20 cm^2 plates were prepared respectively as detailed above (Section 2.5.1) with proportionately larger volumes of components. The agar plates were dried at 42 °C for several hours prior to plating of the H-top mixture; this prevented streaking of the lawn by condensation and the lifting of the H-top layer during subsequent manipulations.
Nylon filters were lowered onto the plaque containing bacterial lawns and a needle was passed through the filter into the agar and an asymmetrical pattern generated that would allow subsequent realignment of the filter with the plate. After 30 seconds the filter was removed and the process repeated with a second filter onto which also was duplicated the asymmetrical pattern; this filter was removed from the surface of the plate after 60 seconds. The nylon membranes were placed, bound DNA side up, on Whatmann 3MM paper soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 1 minute, neutralising solution for 2 minutes (1.5 mM NaCl, 0.5 M Tris.HCl [pH 7.4]) and allowed to air dried. The DNA was cross-linked to the nylon membrane by ultra-violet irradiation (254 nm) for 30 seconds using a Stratalinker 2400.
The membranes were probed with a radioactively labelled DNA fragment, washed (Section 2.12.2), and exposed to X-ray film (Section 2.13). The area of the bacterial lawn seen to contain a plaque hybridising to the radiolabelled probe on both membranes was excised from the plate using a glass pipette and the agar plug placed into SM. The resulting recombinant-bacteriophage stock was titrated and the whole procedure repeated until single clonal bacteriophage, which hybridised with the radiolabelled probe, had been isolated.

Section 2.5.3: Isolation of cloned recombinant-bacteriophage λ DNA

This procedure was necessary for bacteriophage λ clones in EMBL3 and gt11 vectors. 1×10^{10} cells (OD_{600} of 1 represents 8×10^8 cells/ml) from a fresh overnight culture of the appropriate bacterial host were added to 5×10^7 pfu/ml of clonal recombinant-bacteriophage in a final volume of 500 µl and allowed to adsorb for 20 minutes at 37 °C; 10 fold titrations of the bacteriophage stocks were also adsorbed to host cells in this manner. The mixture was added to 50 ml of pre-warmed L-broth and grown overnight at 37 °C in an orbital shaker. At the correct host: bacteriophage ratio a large amount of bacterial debris occurs as the bacteriophage lyse the host cells. 500 ml

cultures were established using the bacteriophage: host cell ratio generating the most lysis. Following over night culturing the remaining intact host cells were lysed by addition of 1 ml of chloroform and shaken for 10 minutes. Bacterial debris was removed from the culture by centrifugation at 5,000 g for 5 minutes and the supernatant decanted. The recombinant-bacteriophage were pelleted from the supernatant by centrifugation at 100,000 g for 90 minutes and the pellet from each 500 ml culture was resuspended in 1 ml of SM. 1 µl of DNase (10 mg/ml) and RNase A (10 mg/ml) were added and the bacterial RNA and DNA digested at 37 °C for 1 hour; the bacteriophage DNA is resistant to these enzymes as it is within the phage coat proteins. This reaction was stopped by addition of 200 µl of TES (0.3 M Tris.HCl (pH 9.0), 0.15 M EDTA and 1.5% SDS [w/v]) and heated at 70 °C for 15 minutes; 150 µl of 8 M potassium acetate was added and the sample left on ice for 15 minutes. The protein was removed from the DNA by phenol extraction and ethanol precipitation (Section 2.11.1) and the DNA resuspended in 100 µl of water.

Section 2.5.4: Isolation of recombinant λZAP II DNA by *in vivo* excision

The recombinant cDNA fragment in λZAP II is flanked by the dissected filamentous bacteriophage (f1) origin of replication (OR) present within the pBS SK II (-) phagemid with the initiation and termination elements of the f1 OR on either side of the these sequences. Host XL1-Blue *E.coli* are grown to an OD_{600} of 1 and mixed with 1 X 10^5 pfu/ml λZAP II SM stock and 1 X 10^3 pfu/ml of the R408 filamentous f1 "helper phage" (Stratagene) to a final volume of 500 µl and allowed to adsorb at 37 °C for 20 minutes. The mixture was added to 5 ml of L-broth and incubated at 37 °C for 3 hours in an orbital shaker. During this period the host cells become infected by both the λZAP II bacteriophage and the f1 R408 filamentous bacteriophage. Proteins produced by the f1 R408 helper phage recognise the f1 OR initiation site in the λZAP II genome and nick it at these sites leading to the production of a single-stranded (ss) copy of the DNA downstream of these sites, which includes the cDNA insert and the pBS SK II (-) sequence, until the termination signal in the f1 OR is reached. The ssDNA molecules are circularised by other f1 encoded proteins leading to the re-formation of functional ORs, the DNA is packaged in coat proteins and secreted from the cell. The cultures were heated at 70 °C for 20 minutes to kill the host bacteria which were removed by centrifugation at 12,000 g for 1 minute. 20 µl of the recombinant packaged form of the pBS SK II (-) phagemid was used to re-infect 200µl of XL1-Blue *E.coli* (OD_{600} of 1) as described above, and plated onto LB plates containing ampicillin; on re-infection a double-stranded DNA phagemid molecule is generated in the ampicillin resistant bacterial colonies formed.

Section 2.6: Cloning in the filamentous bacteriophage M13 and isolation of single-stranded DNA

In order to prepare single-stranded (ss) DNA for sequencing and site-directed mutagenesis, recombinant filamentous bacteriophage M13 (M13) was employed. Subcloning into this vector was as described for general ligation reactions (Section 2.11.2) using the double-stranded replicative form (RF) of M13.
M13 ligation mixes were transformed into competent host cells (JM109 and TG1, Section 2.11.6) and plated in H-top (Section 2.5.2); M13 allows α-complementation to be applied to determine recombinant plaques on addition of 40 µl of X-gal (20 mg/ml in dimethylformamide) and 4 µl of IPTG (200 mg/ml stock in water) to the H-top (Section 2.11.7a).
Using the tip of a sterile toothpick, a single M13 plaque was picked and transferred to 1.5 ml of 2 X TY containing a 25 µl aliquot of fresh host cells at an OD_{600} of 1. The cells and M13 plaque were incubated for 6 hours at 37 °C in an orbital shaker during which period ssDNA bacteriophage particles are extruded from the host cells. The host cells are removed by centrifugation at 12,000 g for 10 minutes and the ssDNA particle-containing supernatant removed; the bacterial pellet was retained to allow preparation of the double-stranded RF M13 (Section 2.3.4). 1 ml aliquots of the supernatant were mixed with 200 µl of PEGS (20% (w/v) polyethylene glycol (PEG 800, Sigma) in 2.5 M NaCl) and placed on ice for 30 minutes; this leads to aggregation of the phage particles which were recovered by centrifugation at 12,000 g for 30 minutes. The PEGS was removed by aspiration and the pellet resuspended in 100 µl TE (pH 8.0). DNA was extracted by phenol extraction and ethanol precipitation (Section 2.11.1), resuspended in 10 µl of water and the DNA concentration estimated (Section 2.3.6).

Section 2.7: Isolation of single-stranded DNA from phagemid vectors

By super-infecting bacterial host cells harbouring the recombinant phagemid with the f1 helper R408 (Section 2.5.4) particles containing ssDNA were produced and DNA siolated as described for M13 ssDNA isolation (Section 2.6).

Section 2.8: DNA Sequencing and computer analysis of DNA, RNA and predicted amino acid sequences

Section 2.8.1: Sequencing single-stranded DNA templates

Single-stranded DNA templates were sequenced using a modified version of the chain-termination method (Sanger *et al.*, 1977) using the Sequenase enzyme (USB), a genetic variant of the T7 DNA polymerase lacking 3' - 5' exonuclease activity.
2 µg of ssDNA was mixed with 1pmole of primer to a final volume of 8 µl in water and 2 µl of sequencing buffer was added (200 mM Tris.HCl (pH7.5), 100 mM $MgCl_2$, 250 mM NaCl). The sample was heated at 65 °C for 2 minutes and allowed to cool to room temperature over a 30 minute period during which time the primer annealed to its complimentary sequence in the template. Labelled DNA was generated from the annealed primer in the extending reaction for 5 minutes at room temperature:

Annealed template/ primer mix	10 µl
0.1 M dithiothrietol	1 µl
5 X Labelling mix diluted 1: 5 in water	2 µl
[α–^{35}S] dATP sp. activity 1000 Ci/mmol 10mCi/ml	1 µl
Sequenase (USB)diluted 1: 8 in dilution buffer	2 µl

5 X labelling mix: 7.5 mM each dCTP, dGTP, dTTP

Sequenase dilution buffer: 10 mM Tris.HCl (pH 7.5), 5 mM dithiothrietol, 0.5 mg/ml BSA

3.5 µl aliquots of the extension reaction were terminated following addition to 4 separate tubes containing 2.5 µl of 8 µM each ddNTP. The termination reaction was incubated for 5 minutes at 37 °C and was stopped by addition of 4 µl of loading buffer (95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, 0.05% Xylene Cyanol FF). The samples were heated at 80 °C for 2 minutes and resolved on a 6% acrylamide, 7 M urea (Ultrapure), 1 X TBE gel. Following electrophoresis the gel was fixed in 10% (v/v) acetic acid, 10% (v/v) methanol and dried under vacuum at 80 °C prior to exposure to X-ray film (Section 2.13).

Section 2.8.2: Sequencing double-stranded DNA templates

Section 2.8.2a: Manual sequencing of double-stranded DNA templates

Double-stranded (ds) DNA templates were prepared using a Qiagen column (Section 2.3.4b). 10 µg of dsDNA in a final volume of 18 µl of water were denatured following addition of 2 µl of freshly prepared 2 M NaOH and incubation for 5 minutes. The sample was neutralised by addition of 8 µl 5 M ammonium acetate (pH 7.5) and, following addition of 100 µl of ethanol, the DNA was precipitated at -70 °C for 20 minutes. The dsDNA was collected by centrifugation at 12,000 g for 20 minutes and resuspended in 7 µl of water. The dsDNA was sequenced exactly as described for ssDNA templates following the addition of 1 pmole of primer (Section 2.8.1).

Section 2.8.2b: Automated sequencing of double-stranded DNA templates

Performed using an Applied Biosystems Model 373A automatic sequencer according to the manufacturer's instructions in collaboration with Mr R. McDonald, Jackson Foundation, Rockville, MD.

Section 2.8.3: Computer analysis of DNA, RNA and predicted amino acid sequence data

Sequence data was analysed for the generation of contiguous DNA sequences, the presence of restriction endonuclease sites, and levels of homology to other specific DNA sequences using the Best, Map and Gap programs of the GCG package respectively (Devereux *et al.*, 1984).Transcription factor consensus binding sequences were defined using the MacPattern package and manually using the GCG Best programme. RNA folding was performed using the Fold and Stemloop (Zucker, 1989) programmes of the GCG package. Protein sequence alignment and phosphorylation site analysis was performed using the Gap and Best programmes of the GCG package. Hydrophobicity and sequence alignment plots of amino acid sequences were generated using the GeneJockey package (Biosoft).

Section 2.9: Generation of cDNA mutants by site-directed mutagenesis

Filamentous bacteriophage M13 ssDNA was prepared from an *E. coli* BM313 *dut*$^-$, *ung*$^-$ host as described (Section 2.6). The *dut*$^-$ mutation leads to a high intracellular concentration of dUTP and several molecules are incorporated per ssDNA molecule; the *ung*$^-$ mutation means that the uracil *N*-glycosylase which normally removes dUTP from the ssDNA template is inactive. The mutagenic oligonucleotides were phosphorylated using T7 polynucleotide kinase (Section 2.12.1b) and the efficiency established by a concomitant radiolabelling phosphorylation reaction; the reaction was stopped by phenol extraction and ethanol precipitation (Section 2.11.1). 10pmoles of the phosphorylated mutagenic oligonucleotide, 10pmoles of non-phosphorylated "universal" oligonucleotide primer (USB) were annealed to 1 µg of dUTP containing ssDNA (Section) following addition of 1 µl of 10 x PE1 buffer (200 mM Tris.HCl (pH 7.5), 100 mM $MgCl_2$, 500 mM NaCl, 10 mM dithiothrietol) in a final volume of 10 µl in water. Second DNA strand synthesis primed from the annealed oligonucleotides was carried out overnight at 16 °C:

10 x PE2 buffer	1 µl
2 mM each dNTPs (Pharmacia)	1 µl
10 mM dATP	1 µl
Oligonucleotide annealed ssDNA template	10 µl
T4 DNA ligase	5 units
Klenow fragment	2 units
	Water to 20 µl

10 x PE2 buffer: 200 mM Tris.HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM dithiothrietol

The sample was transformed into competent JM109 *E. coli* which has a functional uracil *N*-glycosylase leading to destruction of the parental ssDNA strand and replication of the mutated DNA (Kunkel, 1985). ssDNA was isolated and the presence of the mutation confirmed by sequencing (Section 2.8.1).

Section 2.10: The Polymerase Chain Reaction (PCR)

Section 2.10.1: PCR involving recombinant vector DNA template

For reactions involving purified phage, plasmid, or phagemid DNA as a template, the following mixture was prepared:

Template DNA	1 µg
dNTPs (2.5 mM each dNTP [Pharmacia])	8 µl
Oligonucleotide primers (1 µg/µl)	1 µl each
10 X reaction buffer	10 µl
Taq DNA dependent DNA polymerase ("polymerase")	4 units
	Water to 100 µl

10 X reaction buffer: 10 mM Tris.HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin

The mixture was overlaid with 50 µl of white mineral oil (Sigma) to prevent evaporation and subjected to the following reactions in a Hybaid Omnigene:

Mixing, 92 °C	5 minutes	(1 cycle)
Denaturing, 92 °C	30 seconds	(30 cycles)
Annealing, 50 °C	45 seconds	(30 cycles)
Extending, 72 °C	(See below)	(30 cycles)
Terminating, 72 °C	5 minutes	(1 cycle)

The length of the extension step was varied according to the expected length of the product with an extension time of 1 minute per 1Kbp (Innis & Gelfand, 1990).

Section 2.10.2: PCR involving Embyronic stem cell genomic DNA template

For reactions involving Embryonic stem (ES) cell genomic DNA (Section 2.23.8) the titrations of reaction components revealed the optimal conditions for PCR reactions were found to be as detailed above (Section 2.10.1) with some modifications. DMSO was added to 5% (v/v) and the $MgCl_2$ concentration in the reaction buffer was lowered to 10 mM, the reaction mixture was subjected to 60 cycles, as detailed above, with an extension time of 1.5 minutes. After 30 cycles an additional 4 units of *Taq* polymerase was added before completion of the final 30 cycles.

Section 2.11: DNA fragment subcloning and bacterial transformation

Section 2.11.1: DNA fragment isolation and preparation for subcloning

The DNA fragment ("insert" DNA) to be subcloned, whether derived from a plasmid, phagemid, recombinant-bacteriophage or polymerase chain reaction (PCR), was separated from other DNA material on a 1% low-melting point agarose gel. The gel was stained with ethidium bromide and the portion of the gel containing the fragment was dissected from the body of the gel using a scalpel. The insert DNA was isolated from the agarose using either Geneclean (Bio 101 inc.) glass bead solution or GlassMax (BRL) glass bead spun-columns according to the manufacturer's instructions. If the insert DNA required further modification prior to subcloning, for example "end-filling" (Section 2.11.3) or restriction endonuclease digestion (Section 2.3.7), these manipulations were performed. Following these manipulations the enzymes were removed from the insert DNA by mixing the reaction sample with an equal volume of phenol (equilibrated with TE [pH8.0]) and centrifugation at 12,000 g for 5 minutes to separate the aqueous and phenol layers. The upper DNA containing aqueous layer was removed and mixed with an equal volume of chloroform/iso-amyl alcohol (24:1 [v/v]) to remove the last traces of phenol and the phases separated following centrifugation at 12,000 g for 5 minutes. The upper aqueous layer was removed and DNA precipitated for 30 minutes at -20 °C on addition of 0.1 volumes of sodium acetate (pH 4.8) and 2.5 volumes of ethanol; the DNA was collected by centrifugation at 12,000 g for 20 minutes and resuspended in 10 µl of water. Collectively this procedure is subsequently referred to as "phenol extraction and ethanol precipitation"; the DNA concentration was then estimated (Section 2.3.6).

Section 2.11.2: Vector preparation for the receipt of DNA inserts

The vector (phagemid, plasmid or bacteriophage) into which the DNA insert was to be subcloned was digested with the appropriate restriction endonucleases and subjected to any further necessary modifications such as "end-filling" (Section 2.11.3) or dephosphorylation (Section 2.11.4). Following these manipulations the vector was phenol extracted, ethanol precipitated, resuspended in water and the DNA concentration was estimated (Section 2.3.6).

Section 2.11.3: "End-filling" recessed 3' terminals with the "Klenow" fragment

In order to generate DNA molecules with non-recessed 3' termini ("blunt"), from the products of a restriction endonuclease reaction generating recessed 3' termini, the recessed termini are filled by the action of the large fragment of *E. coli* DNA polymerase I ("Klenow" fragment) (BCL). 10 µg of DNA were mixed with 50 mM Tris. HCl (pH7.5), 10 mM $MgCl_2$, 0. 25 mM each dNTP (Pharmacia) and 1 unit of Klenow fragment in a final volume of 25 µl and incubated at 37 °C for 30 minutes. The Klenow fragment was removed by phenol extraction and ethanol precipitation (Section 2.11.1) and the DNA resuspended in 10 µl of water.

Section 2.11.4: Dephosphorylation of 5' terminal phosphates

Vectors linearised using a single restriction endonuclease may recircularise on ligation; this possibility was reduced by removal of the 5' terminal phosphates using calf intestinal phosphatase (CIP) (Promega). 10 µg of DNA was resuspended in 40 µl of 10 mM Tris. HCl (pH 8.0) and 5 µl of 10 x CIP buffer (0.5 mM Tris. HCl (pH 9.0), 10 mM $MgCl_2$, 1 mM $ZnCl_2$, 10 mM spermidine) and 0.5 units of CIP were added and incubated at 37 °C for 30 minutes when a second 0.5 unit addition of CIP was made. After an additional 30 minutes the sample was phenol extracted, ethanol precipitated (Section 2.11.1) and resuspended in 10 µl of water.

Section 2.11.5: Ligation of the prepared insert and vector DNA

For the ligation of DNA fragments and vectors with cohesive terminals 100-200ng of vector DNA and a 5 fold excess of insert were mixed to a final volume of 7.5 µl in water. To this was added 1 µl of 10 x ligation buffer (200 mM Tris.HCl (pH 7.6), 50 mM $MgCl_2$, 50 mM dithiothrietol), 1 µl of 5 mM ATP and 1 unit of T4 DNA ligase (BCL). The reaction was allowed to proceed for no longer than 16 hours at 12 °C. Ligation of DNA fragments and vectors with blunt-ended terminals was carried out as above but without the addition of 5 mM ATP.

Section 2.11.6: Transformation of bacterial cells with plasmid or phagemid DNA =

Section 2.11.6a: Generating transformation-competent bacteria using calcium chloride

1 ml of an overnight *E.coli* culture was added to 40 ml of L-broth and was grown with shaking until the cells reached an OD_{600} of 0.6. Cells were pelleted by centrifugation at 5,000 g, 4 °C for 5 minutes, resuspended in 20 ml of 100 mM calcium chloride, and kept on ice for 30 minutes. The cells were pelleted again by an identical centrifugation step and resuspended in 4 ml of 100 mM calcium chloride. Cells were stored at 4 °C and used within 2 days of preparation.
DNA (0.01 µg-0.10 µg) was added to 200 µl of competent cells and left on ice for 1 hour. The cells were heat shocked at 42 °C for 90 seconds and added to 200 µl of pre-warmed L-broth. The cells were incubated at 37 °C for 1 hour to allow expression of resistance proteins. The cells were pelleted by centrifugation at 12,000 g for 15 seconds, resuspended in 100 µl of water and applied to L-broth plates containing the appropriate antibiotic and detection chemicals, inverted, and incubated overnight at 37 °C.

Section 2.11.6b: Transformation of bacteria by electroporation

$CaCl_2$ treatment does not generate a high level of transformation competent cells in *S. typhimurium* strains. These bacteria were transformed by electroporation; 0.1 µg of DNA was mixed with 500 µl of cells with an OD_{600} of 0.6. The cells were electroporated at 40 µF, 0.2 kV using an IBI electroporator. The cells were treated as detailed above (Section 2.11.6a) from the heat shock treatment at 42 °C.

Section 2.11.7: Screening of bacterial colonies transformed with recombinant plasmid or phagemid constructs

Section 2.11.7a: α-Complementation screening of bacterial colonies

The pTZ18R and 19R and pUC18 and 19 vectors used in this study carry a short segment of DNA that contains the regulatory sequence and the first 146 amino acids of the *E. coli lac Z* gene encoding the β-galactosidase enzyme. On transformation of the parent plasmid into certain host strains, which produce the C-terminus of the β-galactosidase enzyme encoded on the F' episome, the two fragments can interact and produce an active protein (Ullman *et al.*, 1967); this process is termed α-

complementation. Bacteria transformed with recombinant vector form blue colonies in the presence of X-gal (5-bromo-4-chloro-3-indolyl-β- D-galactoside) on derepression of the *lac Z* gene fragments by IPTG (isopropylthio-β-D-galactoside) addition. Insertion of the recombinant fragment into the cloning site of the vector however disrupts the lac Z N-terminal fragment and colonies containing recombinant plasmids are white.

Bacteria transformed with vectors allowing α-complementation to be performed were plated onto L-agar, ampicillin plates onto which was spread 40 µl of X-gal, from a stock of 20 mg/ml in dimethylformamide, and 4 µl of IPTG, from a 200 mg/ml stock in water.

Section 2.11.7b: Screening of bacterial transformants by colony hybridisation

If α-complementation was not possible due to the nature of the construct transformed, a modification of the method of Grunstein and Hogness (1975) was used to screen for recombinant bacteria. Transformed colonies resulting were picked using sterile toothpicks and duplicate colonies streaked onto Hybond-N gridded nylon membranes (Amersham) placed on L-agar plates containing ampicillin. Plates were inverted and incubated at 37 °C overnight. One plate was stored at 4 °C (the "master" filter); the second filter was removed from the L-agar plate and placed colony side up on Whatman 3MM paper soaked in 10% SDS (w/v) for 3 minutes, denatured for 5 minutes (0.5 M NaCl, 1.5 M NaOH) and neutralised for 5 minutes (0.5 M Tris-HCl (pH.7.5), 1. 5 M NaCl). Filters were air dried at room temperature and baked at 80 °C for 2 hours. The bacterial debris was removed from the filters by washing in 50 mM Tris. HCl (pH 8.0), 1.5 M NaCl, 1 mM EDTA, and 1% SDS (w/v) in a shaking water bath at 42 °C for 1 hour. Filters were prehybridised and hybridised with radiolabelled insert containing probes, washed and exposed to X-ray film (Sections 2.12.2 and 2.13). Recombinant colonies seen to hybridise with the probe were picked and grown from the duplicate colony on the retained master filter.

Section 2.12: DNA and RNA hybridisation techniques

Section 2.12.1: Radiolabelling DNA probes

Section 2.12.1a: Random prime labelling of double-strand derived DNA probes

Double-stranded DNA was labelled by the random priming method of Feinberg and Vogelstein (1983). The DNA insert was heat denatured in the presence of a molar excess of random hexanucleotide primers and radioactive nucleotides were added into the probe DNA following primer extension by the large fragment of *E. coli* DNA polymerase I ("Klenow" fragment). 25 ng of the DNA to be labelled was denatured at 100 °C for 3 minutes and mixed with the following components; the labelling reaction was allowed to proceed at 37 °C for at least 3 hours:

OLB	3 µl
BSA (10 mg/ml, Sigma)	2 µl
DNA	25 ng
[α-^{32} P] dCTP sp. activity >3, 000 Ci/mmole; 10 Ci/ml	3 µl
Klenow fragment	2 units
	water to 100 ul

OLB comprised of a mixture of the solutions A, B, and C in a ratio of 2: 5: 3:

A:	2 M Tris-HCl, pH 8.0	625 µl
	5 M $MgCl_2$	25 µl
	Water	350 µl
	2-mercaptoethanol	19 µl
	100 mM dTTP, dCTP, dGTP, and dATP in 3 mM Tris-HCl (pH 8.0) 0.2 mM EDTA (Pharmacia)	5 µl of each

B: 2 M HEPES (pH 6.6)

C: Hexadeoxyribonucleotides in 3 mM Tris. HCl (pH8.0), 0.2 mM EDTA (pH 7.0) to a concentration of 9000 units/ml (Pharmacia).

Section 2.12.1b: Radiolabelling oligonucleotide probes

Synthetic oligonucleotides are synthesised without a phosphate group at the 5' terminal and can be labelled by the transfer of [γ-^{32}P] ATP to the 5' termini using the bacterial T4 polynucleotide kinase (PNK) in the following reaction mixture; the labelling reaction was allowed to proceed at 37 °C for at least 45 minutes:

Oligonucleotide (10 pmoles/µl)	1 µl
10 X PNK buffer	2 µl
[γ-^{32}P] ATP spec. activity 5, 000 Ci/mmole. 10 Ci/ml	5 µl
PNK (10 units)	1 µl
Water	11 µl

10 X PNK buffer: 0.5 M Tris.HCl (pH 7.6), 0.1 M $MgCl_2$, 50 mM dithiothrietol, 1 mM spermidine, 1mM EDTA (pH 8.0)

Section 2.12.1c: Establishing the efficiency of radiolabel incorporation into both oligonucleotide and double-strand DNA derived probes

1 µl of the labelled probe mixture was spotted onto Whatman DE-81 filter paper. The filter is positively charged and binds oligonucleotide and double-stranded DNA to the origin whereas unincorporated radiolabelled nucleotides are bound less strongly and can be separated chromatographically using 0.3 M ammonium formate (pH 8.0). By exposing the resultant filter to X-ray film an assessment of the relative levels of incorporated to unincorporated radiolabelled nucleotides was obtained; an efficiency of greater than 80% was routinely obtained.

Section 2.12.2: Hybridisation of radiolabelled double-strand DNA derived DNA probes to membrane-bound DNA

Membrane-bound DNA samples were prehybridised for at least 3 hours at 65 °C either in sealed plastic bags in a shaking water bath or in glass tubes in a rotisserie oven (Techne).

Prehybridisation solution, prepared to a final concentration in water of:

5 X SSPE
0.1% Sodium pyrophosphate (w/v)
0.5% SDS (w/v)
100 µg/ml heat denatured and needle sheared herring sperm DNA
2 X Denhardt's solution

20 X SSPE (pH 8.0) /litre: 175.3 g NaCl, 27.6 g NaH_2PO_4, 7.4 g EDTA
50 X Denhardt's solution/litre: 10 g each of BSA (Sigma), polyvinylpyrollidine (Sigma), Ficoll-400 (Sigma) to 1 litre with water.

Heat denatured and needle sheared herring sperm DNA (Sigma) was diluted from a 10 mg/ml stock.

Hybridisation solution: As for prehybridisation solution but without the inclusion of herring sperm DNA

The radiolabelled insert DNA probe was denatured at 100 °C for 3 minutes, added to the filter and hybridised at 65 °C overnight.

Washing conditions: All washes were carried out at 65 °C with solutions heated to 65 °C prior to addition. Each wash was carried out for 20 minutes in a progressively lower salt solution as indicated below. After the 1 x SSC wash the level of background radioactivity attached to the filter was assessed using a hand-held monitor and the lengths of the subsequent lower salt washes varied accordingly.

2 X SSC, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)
1 X SSC, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)
0.2 X SSC, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)
0.1 X SSC, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)

20 X SSC (pH 7.0): 175.3 g NaCl, 88.2 g sodium citrate.

Section 2.12.3: Hydridisation of radiolabelled oligonucleotide probes to membrane-bound DNA

Filters were prehybridised for at least 3 hours at 42 °C in glass tubes in a rotisserie oven (Techne) :

Prehybridisation solution to a final concentration in water of:

6 X SSPE
5 X Denhardt's solution
0.5% SDS (w/v)
0.1% sodium pyrophosphate (w/v)
100 µg/ml heat denatured and needle sheared herring sperm DNA

Hybridisation solution: As for prehybridisation solution without the inclusion of herring sperm DNA.

Washing conditions: Filters were washed in pre-warmed 6 X SSC, 0.1% Sodium pyrophosphate (w/v) at 48 °C for 30 minutes. Filters were examined with a hand-held monitor and if they exhibited high levels of background radioactivity they were washed in the same washing solution but with progressive 4 °C increases in temperature.

Section 2.12.4: Hybridisation of double-stranded DNA derived probes to membrane-bound RNA

Membrane-bound RNA samples were prehybridised for at least 3 hours at 42 °C in glass tubes in a rotisserie oven (Techne). The double-stranded DNA derived probe was denatured at 100 °C for 3 minutes, added to the membrane and hybridised over night at 42 °C.

Pre-hybridisation/ hybridisation solution:

100% Formamide	10 ml
50 X Denhardt's solution (Section 2.12.2)	2 ml
10% (w/v) SDS	1 ml
20 X SSC (Section 2.12.2)	6 ml
50% (w/v) Dextran sulphate	3 ml
100 µg/ml heat denatured and needle sheared herring sperm DNA	200 µl

Washing conditions: All washes were carried out at 65 °C with solutions pre-warmed to 65 °C as described in Section 2.12.2.

Section 2.13: Autoradiography

For analysis of filter-bound [γ-^{32}P] ATP, [α-^{32} P] dCTP and I^{125}: After washing, filters were wrapped in clear plastic film (Saran-wrap) and exposed to Kodak X-AR film at -70°C in X-ray cassettes. Intensifying screens were incorporated in the cassettes containing filters binding [γ-^{32}P] ATP and [α-^{32} P] dCTP.

For analysis of [α-^{35}S]-dATP containing gels and ^{14}C containing TLC plates: after drying the radioactive materials were exposed directly to Kodak X-ARS film in X-ray cassettes at room temperature ([α-^{35}S]-dATP) and -70 °C (^{14}C).

Films were developed using a Geramatic 60 (Agfa-Gevaert) automatic developer.

Section 2.14: Heterologous expression of mammalian P-450 in bacteria

Section 2.14.1: IPTG (isopropylthio-β-D-galactoside) derepression of bacterial hosts harbouring pKK223-3(mod) and pET15b constructs

Host cells (*E. coli* and *S. typhimurium*) were grown to an OD_{600} of 0.5 at 28 °C with the relevant antibiotics (50 μg/ml ampicillin in both cases and for pET15b additionally 25 μg/ml chloramphenicol). IPTG was added to a final concentration of 1 mM and the culture was grown for 6 to 8 hours at 28 °C. Prior to induction δ-amino laevulinic acid (Sigma) was added to a final concentration of 6×10^{-6} M in some instances.

Section 2.14.2: Small scale isolation of protein from IPTG-derepressed bacteria

Derepressed host cells were collected by centrifugation at 5,000 g for 5 minutes and resuspended in sucrose KCl-phosphate buffer (Section 2.19). The cells were disrupted following two 15 second sonication steps at full power (Soniprep 150 sonicator, MSE); during these manipulations the samples were kept on ice. The protein concentration in the samples was estimated (Section 2.20).

Section 2.14.3: Isolation of subcellular fractions of IPTG-derepressed bacteria

Based on the method of Yoshida and Aoyama (1984). Host cells were collected by centrifugation at 5,000 g for 5 minutes and resuspended in sucrose KCl-phosphate buffer (Section 2.19); the cells were disrupted by passage through a French press (Enerpak) and the ice at a pressure of 200 lb/inch^2; samples were kept on ice before and after this procedure. Cell debris and unbroken cells were removed by centrifugation at 5,000 g for 5 minutes and a membrane enriched fraction was isolated by centrifugation of the supernatant for 2 X 80 minutes at 100,000 g. The membrane enriched pellet was resuspended in sucrose KCl-phosphate buffer using a hand-held teflon homogeniser between centrifugation steps. The cytosolic fraction from the first ultracentrifugation step was retained and used as a source for reducing equivalents in *p*-nitrophenol assays (Barnes *et al.*, 1991. Section 2.14.5). Protein concentrations of the fractions were estimated (Section 2.20).

Section 2.14.4: Measurement of P-450 haemoprotein by difference-spectra analysis

Performed according to the method of Omura and Sato (1964). A known concentration of protein was added to cuvettes in a final volume of 1 ml in KCl-phosphate buffer (Section 2.19) and an absorbance base line established between 400 and 500 nm on a Shimadzu MPS-2000 scanning spectrophotometer. A few grains of sodium dithionite were added to the cuvettes to reduce the P-450 and carbon monoxide was bubbled through one cuvette. The difference spectra between the two samples was recorded between 400 to 500 nm; the presence of detectable levels of haem within the P-450 generated an absorbance peak at 450 nm.

Section 2.14.5: *p*-Nitrophenol hydroxylation as a probe for CYP2E subfamily activity

The hydroxylation of *p*-nitrophenol correlates with the observed level of CYP2E subfamily protein in assays performed using perfused livers, purified CYP2E protein and microsomal material and the activity is inhibited (>95%) following addition of CYP2E subfamily inhibitory antibodies (Reinke & Moyer, 1985. Koop, 1989). *p*-Nitrophenol hydroxylation generates a 4-nitrocatechol product which can be detected by an increased absorption at 510 nm.

Assays were performed in the following incubation mixtures with a known protein concentration in a final volume of 0.6 ml in KCl-phosphate buffer (Section 2.19) :

NADPH regenerating system	0.2 ml
60 μM *p*-nitrophenol	0.2 ml
Protein fraction	0.6 ml

NADPH regenerating system: 4 μmoles glucose 6-phosphate, 0.4 μmoles NADP, 0.4 units glucose 6-phosphate dehydrogenase, 2 μmoles $MgCl_2$.

In assays of bacterial fractions cytosolic protein (Section 2.14.3) was added in an equal protein amount to the assayed protein concentration, to provide reducing equivalents for the reaction. Studies have shown that this fraction contains components of an electron transport system that are capable of donating electrons to mammalian P-450 and so substituting for the P-450 reductase (Barnes *et al.*, 1991). The constituents were incubated at 37 °C in a shaking water bath and the reaction was initiated following addition of the substrate. The reaction was allowed to proceed for 12 minutes and was stopped by addition of 0.25 ml of 0.6 M perchloric acid and centrifuged for 5 minutes at 12,000 g. 1 ml of the supernatant was added to 0.1 ml of 10 M NaOH to ionise the 4-nitrocatechol and the absorbance at 510 nm was read immediately in a Shimadzu MPS-2000 scanning spectrophotometer.

Section 2.15: Manipulation and culturing of *Saccharomyces cerevisiae*

Section 2.15.1: *S. cerevisiae* ("yeast") strains used in this study

KY118: *a, his* 3-200,*lys* 2-801^{am},*ade* 2-101^{oc},*trp*D1, *ura* 3-52

Used for expression studies as a host for the pMA56, pVT100U and YepGAL vectors

W3031B: *a, leu* 2, *his* 3, *trp* 1, *ura* 3, *ade* 2-1, can^{R}, cyr +

Used for expression studies as a host for the pYeDP1-10 vector

Section 2.15.2: *S. cerevisiae* culture media

Yeast culture media/litre: (YPD)	10 g yeast extract, 10 g peptone (Difco), 20 g glucose, 20 mg Adenine sulphate (Sigma)
YPD agar/litre:	As above with an additional 20 g agar
Minimal media/litre : (YMM)	6.7g yeast nitrogen base without amino acids (Difco), 20 g glucose, 20 mg each amino acid and vitamin supplement required to give the requisite minimal media dependent on the strain/selection employed
Supplemented YMM/litre: (SYMM)	As above with an additional 10 g casamino acids
SYMM agar/litre:	As above with an additional 20 g agar

Section 2.15.3: The transformation of DNA constructs into *S. cerevisiae* using the lithium acetate method

S. cerevisiae were made competent for DNA transformation by a modification of the lithium acetate method of Itoh *et al.* (1983). A culture of the strain to be made competent was grown at 28 °C in YPD until the OD_{600} reached 0.4. (early log. phase). The cells were harvested by centrifugation at 5,000 g for 5 minutes and resuspended in TE (pH 8.0). Lithium acetate (Sigma) was added to a final concentration of 0.1 M and the cells were incubated with gentle shaking at 30 °C for 1 hour. DNA (10 µg) was added to 200 µl aliquots of the cells and the mixture incubated for 30 minutes. Following this incubation PEG-4000 (Sigma) was added to a final concentration of 35% (w: v) and incubated for 1 hour at 30 °C. The cells were heat-shocked at 42 °C for 5 minutes, collected and washed by 2 periods of centrifugation for 2 minutes at 12,000 g followed by resuspension in water and plated onto minimal media plates lacking the appropriate nutrient to allow selection of transformants. The plates were inverted and incubated at 28 °C until transformed colonies appeared.

Section 2.15.4: Small scale isolation of protein from *S. cerevisiae*

Glass beads (40mm mesh) were washed in concentrated HCl and repeatedly rinsed in water until the liquid surrounding the beads reached pH 7.0. At pH 7.0 the beads were washed in KCl-phosphate buffer (Section 2.19) and excess liquid removed using a pipette. Centrifugation harvested *S. cerevisiae* were resuspended in KCl-phosphate buffer and the beads were added to just below the meniscus of the suspension. The mixture was vortexed for 2 X 2 minutes and cooled on ice. The supernatant was removed from the sample and the protein concentration estimated (Section 2.20).

Section 2.15.5: Isolation of *S. cerevisiae* subcellular fractions and the analysis of P-450 difference spectra

As described for the preparation of bacterial subcellular fractions employing a French Press (Section 2.14.3) using a modification of the method of Yoshida and Aoyama *et al.* (1984); an initial centrifugation step at 11,000 g replaced the 5,000 g step however in order to remove the mitochondrial fraction from the supernatant applied to the subsequent ultracentrifugation steps.

Section 2.16: Animal maintenance and manipulation

Unless otherwise stated animals were kept at, and manipulations performed in, the ICRF Clare Hall Laboratories, South Mimms, Hants. Animals were fed a standard laboratory chow diet *ad libitum* and acclimatised for at least 4 days to a 12 hour light/dark cycle prior to treatment.

Section 2.16.1: Mouse lines used in this study

BALB/c: used in cDNA library preparation
C57BL/6: used in regulation studies
DBA2/N: used in regulation studies

Section 2.16.2: Experimental treatment of mice

Section 2.16.2a: Chemical treatment of mice

C57BL/6 and DBA2/N, male and female as stated, were subjected to a 3 day regime of daily intraperitoneal injections (ip) or intragastric (ig) injections of:

Dexamethasone (ip)	100 mg/kg
Pyrazole (ip)	200 mg/kg
N-nitrosodiethylnitrosamine (NDMA) (ip)	200 mg/kg
β-napthaflavone (ip)	80 mg/kg
3-methylcholanthrene (ip)	200 mg/kg
TCPOBOP (ip)	2 mg/kg
2-acetylaminofluorene (ip)	50 mg/kg
Phenobarbital (ig)	80 mg/kg

TCPOBOP: 1,4-bis[2-(3,5-dichloropyridyloxy)benzene]

Animals were also given one dose of aflatoxin β1 (50 mg/kg, ip).
All compounds were administered in corn oil apart from pyrazole and NDMA which were administered in PBS (Section 2.16.5).
Acetone treated animals were administered 1% (v/v) acetone in their drinking water over a 14 day period; animals allowed to recover from this treatment were returned to normal drinking water for a 7 days.

Section 2.16.2b: Starvation of mice

Food and bedding material were withdrawn from animals for the stated periods.

Section 2.16.3: Rat lines used in this study

Wistar: Used for chemical induction studies
BB/E : Used to study the effect of the diabetic state on xenobiotic metabolising systems.

Section 2.16.4: Experimental treatment of rats

Section 2.16.4a: Chemical treatment of Wistar rats

Acetone treatment of rats was identical to that described for acetone treatment of mice (Section 2.16.2a).

Section 2.16.4b: Maintenance and monitoring of the BB/E spontaneously diabetic rat

Animals from the diabetes prone (DP) subline, which show a 55-70% incidence of diabetes, were weighed 3 times a week and the urine of those animals which did not record a weight gain was analysed for the presence of glucose using m-multistix (Bayer diagnostics). In those animals seen to have glucose present in their urine, daily blood glucose analysis was undertaken on tail vein bleeds using BM-test strips (BCL). Animals were classed as diabetic when their blood glucose levels rose above 18 mmoles/litre. One group of diabetic animals detected in this manner were left for 3 days without insulin and sacrificed. A second group of diabetic animals was left without insulin over the same period after which insulin was administered as a single subcutaneous dose (Ultratard Bovine insulin, U-40 strength, ultralente, Novo, Denmark) until the blood glucose level returned to the normal range of 2-4 mmoles/litre. Age- and sex-matched animals from the diabetes resistant (DR) subline, with an incidence of diabetes of less than 1%, were taken for each of the DP animals used in the study.
Animals were kept in isopositive pressure isolators and fed on SDS rat and mouse №1 expanded feed (Special Diet Services, Witham, U.K.). Manipulations and maintenance of the BB/E rats were performed in collaboration with Dr J. D. Baird, Dr R. M. Lindsay and Mr W. Smith, at the Metabolic Unit, University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh.

Section 2.16.5: Animal sacrifice and tissue removal

Animals were killed by cervical dislocation, organs removed, rinsed in phosphate buffered saline (PBS) and frozen in liquid nitrogen. Blood samples were removed and placed in Lithium-heparin anticoagulent tubes. Samples for electron microscopy and immunohistochemistry were placed in 2.5% (v/v) glutaraldehyde in KCl-phosphate buffer (Section 2.19) and formal saline (14% (v/v) formaldehyde in PBS) respectively.

PBS/litre: 1.5 g Na_2HPO_4 , 8 g NaCl, 0.1 g $MgCl_2.6H_2O$, 1.5 g KH_2PO_4, 0.2 g KCl, 0.132 g $CaCl_2.2H_2O$

Section 2.17: Ultrastructural studies of fixed animal tissues

Section 2.17a: Transmission electron microscopy and peroxisome percentage assessment

Liver samples from the BB/E DP and DR animals (Section 2.16.5) fixed in 2.5% (v/v) glutaraldehyde in KCl-phosphate buffer (Section 2.19) were prepared for transmission electron microscopy which was performed using a Jeol 100CX TEM in collaboration with Dr J. Foster and Dr C. Bowling, Zeneca Central Toxicology laboratory, Cheshire. Three representative areas of the liver sample were photographed at X 8,300 magnification and the micrographs were analysed for cytoplasmic peroxisomal content using a 345 intercept square lattice allowing the percentage of the cytoplasm occupied by peroxisomes to be assessed (Moody & Reddy, 1976).

Section 2.17b: Immunohistochemical analysis of animal tissue

Animal tissue fixed in formal saline (Section 2.16.5) were paraffin embedded, sectioned and the structures assessed following staining with haematoxylin, which stains nuclei black, and eosin, which stains cytoplasm and connective tissues red. Fixed sections were immunohistochemical analysed following incubation with antibodies to certain P-450. Tissue sections were blocked in pre-immune rabbit serum (1:5 (v/v) dilution in PBS) for 10 minutes and incubated in the P-450 antibody of interest (1:10 (v/v) dilution in TBS) at 4 °C overnight. The sections were blocked again with pre-immune rabbit antisera as above and incubated with biotinylated swine anti-rabbit serum for 30 minutes. The binding of the first and second antibody complex was visualised following addition of the avadin-biotin horseraddish peroxidase complex and its substrate (0.068% (w/v) imidazole, 2% (v/v) H_2O_2, 0.05% (w/v) 3, 3-

diaminobenzidine) which binds to the immune complex and produces a brown staining. The surrounding tissue structure was stained using haemotoxylin; tissue manipulation and immunostaining was performed in collaboration with Dr D. J. Harrison and Mrs H. Wolf, Department of Pathology, Edinburgh University.

Section 2.18: Biochemical analysis of animal sample material

The following analysis of components of the blood collected from the BB/E rats were performed in collaboration with Dr R. M. Lindsay, The Metabolic Unit, University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh:

1) The percentage glycated haemoglobin content was determined using boronic acid affinity chromatography (Pierce & Warriner).

2) The plasma insulin concentration was measured by radioimmune assay using rat insulin standards (Novo, Denmark) and charcoal separation (Ashby and Speake, 1975).

3) Plasma concentrations of cholesterol and triglycerides were determined colorimetrically on a Kodak 700XR C Series analyser (Kodak Clinical diagnostics).

4) Plasma non-esterified ("free") fatty acid concentration was assessed by an enzymatic colorimetric assay (Wako Chemicals) using a centrifugal fast analyser.

5) Plasma hydroxy butyrate was determined spectrophotometrically using a Ranbut enzymatic kit (Randox).

Glutathione S- Transferase activities in cytosolic fractions were assessed using a 1-chloro-2,4-dinitrobenzene (CDMB) spectrophotometric assay in collaboration with Dr L. I. McClellan, Department of Clinical Chemistry, Royal Infirmary, Edinburgh.

The level of cyanide insensitive β-oxidation, a measure of peroxisomal activity, was analysed in the "heavy pellet" spectrophotometrically in collaboration with Dr C. Elcombe and Dr S. Tittensor, Zeneca Central Toxicology laboratory, Cheshire using the method of Bronfman *et al.* (1979).

Section 2.19: Isolation of subcellular fraction enriched protein samples from mouse and rat tissue

The tissue was added to 3 volumes of KCl-phosphate buffer (10 mM potassium phosphate (pH 7.4), 1.15% (w/v) KCl, 0.1 mM EDTA) and homogenised in a Silverson Laboratory mixer homogeniser at 4 °C. The homogenate was centrifuged at 11,000 g for 20 minutes to remove cell debris and larger cellular organelles such as mitochondria, nuclei and peroxisomes; this fraction constituted the "heavy pellet" and was resuspended in KCl-phosphate sucrose buffer (0.25 M sucrose, 10 mM potassium phosphate (pH 7.4) 1.15% (w/v) KCl, 0.1 mM EDTA). The supernatant was centrifuged at 45,000 g for 80 minutes at 4 °C to pellet the endoplasmic reticulum-enriched "microsomal" fraction. The supernatant from this centrifugation was retained and constituted the cytosolic fraction in susequent analysis. The microsomal pellet was resuspended in KCl-phosphate buffer using a Teflon-glass hand-homogeniser and recentrifuged at 45,000 g for 60 minutes at 4 °C. The resulting pellet was resuspended in sucrose buffer by hand-homogenisation; the protein concentration was estimated (Section 2.20) and samples were maintained at -40 °C.

Section 2.20: Protein concentration estimation

The protein concentration of samples to be analysed was determined by the method of Lowry *et al.* (1951) using a range of known bovine serum albumin (BSA) protein concentrations as standards (0 to 200 µg/ml). The sample to be analysed was mixed 1:40 (v/v) in 0.1 M NaOH. To this was added 5 volumes of freshly prepared alkaline carbonate solution (490 ml 70 mM Na_2CO_3: 40 mM NaOH, 5 ml 40 mM $CuSO_4$, and 5 ml 71 mM potassium tatrate per 500 ml) and the solution was incubated for 10 minutes. 0.1 volumes of Folin Coicalteau reagent was added and the sample incubated for 1 hour. The absorbance at 600 nm was determined using a Shimadzu U.V. 160 spectrophotometer.The standard protein concentration values were determined in duplicate and the sample values in triplicate.

Section 2.21: Analysis of isolated protein fractions

Section 2.21.1: Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS PAGE) of protein samples

Protein samples were prepared for SDS PAGE following dilution to a protein concentration of 4 mg/ml in water, and addition of an equal volume of boiling mix (0.05 M Tris.HCl (pH 6.8), 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue dye); the sample was heated at 100 °C for 5 minutes.

For detection of P-450 proteins (M_r of approximately 50,000) samples were separated on 9% (w/v) polyacrylamide (8.7% acrylamide, 0.3% *N, N'*-methylene-bis-acrylamide) in 0.375 M Tris.HCl (pH 8.8), 0.15% (w/v) SDS. Ammonium persulphate (AMPS, 1% [w/v]) and *N, N, N ', N '*-tetra-methyl ethylenediamine (TEMED) were added to 0.05% (v/v). For detection of lower molecular weight proteins, such as the approximately M_r 20,000 GSTs, samples were separated on 12% (w/v) polyacrylamide gels. A stacking gel was prepared (4.5% (w/v) acrylamide, 0.125 M Tris.HCl (pH 6.8), 0.125% SDS, 0.05% each of TEMED and AMPS) and poured over the the polymerised separating gel. The gels were prepared in BioRad Protean 2 vertical electrophoresis equipment and electrophoresed in electrode buffer (0.05 M Tris.HCl (pH 8.3), 0.05 M glycine, 0.1% SDS). The protein sample was electrophoresed through the stacking gel at 50mA and the separating gel at 30 mA per gel at constant current.

Section 2.21.2: Protein molecular weight standards

Rainbow molecular weight markers (Amersham) were used to estimate the M_r of proteins separated by SDS PAGE:

Protein	M_r
Myosin	200,000
Phosphorylase b	92,000
Bovine serum albumin	69,000
Ovalbumin	46,000
Carbonic anhydrase	30,000
Trypsin inhibitor	21,500
Lysozyme	14,300

Section 2.21.3: Western blot analysis of SDS PAGE separated protein samples

Western blot analysis was performed by a modification of the method of Towbin *et al.* (1979). SDS PAGE separated protein was transferred using a BioRad Protean II transblot cell to 0.45 µm nitrocellulose filters (Schindler and Schuell) in transblot buffer (20 mM disodium orthophosphate, 20% (v/v) methanol) for 12 hours at 250 mA. After protein transfer the filter was washed in TBST (50 mM Tris.HCl (pH 7.9), 0.15 M NaCl, 0.005% (v/v) Tween 20 [Sigma]) and blocked in 3% (w/v) low fat powdered milk (Marvel, Cadbury's) in TBST for 1 hour. After blocking the filter was washed for 2 X 20 minute periods in TBST, and the first antibody, raised against the protein of interest, was added at a 1:500 dilution in TBST and left for 1 hour. The first antibody was removed and the filter washed by 4 X 20 minute periods in TBST and the second antibody was added and incubated for 1 hour. The nature of the first antibody dictated the second antibody added; if the first antibody was a mono- or poly- clonal then rabbit anti-mouse or goat anti-rabbit antibodies were added respectively at a 1: 1000 dilution in TBST. The immunoreactive protein-antibody complexes were iodinated in 0.19 MBq of ^{125}I-conjugated protein A in 50 ml of TBST for 45 minutes followed by repeated washing in TBST to remove non-specifically bound radioactivity. The filters were exposed to X-ray film (Section 2.13). Alternatively the immunoreactive protein-antibody complexes were visualised using the Enhanced Chemiluminescence (ECL) method (Amersham). The second antibodies are labelled with horse-raddish peroxidase (HRP) which catalyses the oxidation of luminol in the presence of H_2O_2 generating light and producing a response on X-ray film (Roswell & White, 1978); the reaction was performed according to the manufacturer's instructions.

Section 2.21.4: Antisera used in the detection of proteins in this study

Polyclonal antisera to purified rat P-450 isoforms, other than CYP2E, have been previously characterised for their specificity towards the stated epitopes (Wolf & Oesch, 1983. Wolf *et al.*, 1984). The CYP2E polyclonal antibody was raised against purified rat protein and was the kind gift of Professor C. S. Yang, Rutgers University, NJ. The specificity of the polyclonal antibodies raised to the rat CYP2E1 protein in the detection of mouse Cyp2e1 has been previously demonstrated (Hong *et al.*, 1989.). Monoclonal antisera raised to purified rat CYP4A1 protein was produced by Ciba-Geigy, Basel, Switzerland and its specificity has been previously demonstrated (Savoy *et al.*, 1990).

Polyclonal antisera to several cytosolic GST subunits and purified glyoxylase I were the kind gift of Dr L. I. McClennan (Department of Clinical Chemistry, Royal

Infirmary, Edinburgh) and their specificity has been previously demonstrated (Hayes & Mantle, 1986). Polyclonal antisera raised against purified rat microsomal GST was the kind gift of Dr M. B. Crichton (ICRF MPG, Edinburgh).

Section 2.22: Isolation and analysis of total RNA from mouse and rat tissues

Section 2.22.1: Isolation of total RNA

Total RNA was isolated from tissue samples for Northern blot analysis using the method of Chomczynski and Sacchi (1987). 0.5 g of tissue was added to 5 ml of solution D (Stock: 250 g guanidinium thiocyanate (Sigma), 293 ml water, 17.6 ml Sodium citrate (pH 7.0), 26.4 ml 10% (v/v) sarcosyl; 0.36 ml of 2-mercaptoethanol was finally added per 50 ml of stock to produce solution D). The tissue was homogenised in solution D using a Silverson Laboratory mixer homogeniser. The homogenate was centrifuged at 9,000 g for 5 minutes to remove insoluble tissue debris and sequentially 0.5 ml of 2 M sodium acetate (pH 4.0), 5 ml of water saturated phenol, and 1 ml of chloroform/isoamyl alcohol (49:1 [v/v]) were added to the supernatant. The sample placed on ice for 20 minutes. Samples were centrifuged at 10,000 g for 20 minutes at 4 °C, RNA was present in the upper aqueous phase which was removed and RNA precipitated at -20 °C for 20 minutes following addition of 5 ml of isopropanol. RNA is collected by centrifugation at 10,000 g for 20 minutes at 4 °C and the pellet resuspended in 1.5 ml of solution D; RNA is reprecipitated from this solution at -20 °C for 1 hour following the addition of an equal volume of isopropanol. The RNA is collected by centrifugation at 12,000 g for 20 minutes, resuspended in 1 ml of water, and heated at 65 °C for 10 minutes. RNA concentrations were then estimated (Section 2.3.6) and the samples stored at -70 °C.

Section 2.22.2: Northern blotting of isolated total RNA

RNA was size fractionated on horizontal denaturing agarose gels; 1% (w/v) agarose was melted in water and allowed to cool to 60 °C before addition of 5 x GRB (0.1 M MOPS (3-[*N*-morpholino]propanesulfonicacid) (pH 7.0), 40 mM Sodium acetate, 5 mM EDTA [pH 8.0]) and 12.3 M formaldehyde to give a final concentration of 1 x and 2.2 M respectively.

RNA samples were prepared following the addition of :

RNA (known concentration in water)	4.5 µl
5 X GRB	2.0 µl
Formaldehyde	3.5 µl
Formamide	10.0 µl

The sample was heated at 55 °C for 15 minutes and 2.0 µl of loading dye (0.4% (w/v) each bromophenol blue and xylene cyanol FF, 50% glycerol, 1 mM EDTA [pH 8.0]) was added prior to loading. 3 µg of RNA size markers (BRL) were treated in the same manner as the RNA sample and loaded onto the gel.The gel was electrophoresed in 1 X GRB and stained with ethidium bromide (Section 2.3.8) to establish the equal loading of the samples by the relative amounts of 28 S (6,333 nucleotides) and 18 S (2,366 nucleotides) rRNA species present in the different tracks. The gel was washed in 10 X SSC (Section 2.12.2) and transferred to hybond-N nylon membranes (Amersham) by capillary transfer in 10 X SSC. The portion of the membrane corresponding to the track on the gel containing the RNA markers was cut off and stained in methlyene blue (0.04% methylene blue (w/v), 0.5 M NaOH) for 20 minutes and washed in several changes of water to reveal the blue stained RNA size standards. The rest of the membrane was treated as described for Southern blotting following capillary transfer of the DNA sample to the nylon membrane (Section 2.3.11).

RNA size markers (Kb): 9.5, 7.5, 4.4, 2.4, 1.4, 0.3

Section 2.23: Mammalian tissue culture manipulations

Section 2.23.1: Cell lines used in this study

COS 7:	Monkey kidney derived (Anderson *et al.*, 1989)
HepG2:	Human hepatocarcinoma derived (Knowles *et al.*, 1980)
$C_3H/10T1/2$:	Mouse embryo derived (Reznikoff *et al.*, 1973)
Hepa 1:	Mouse liver derived (Bernhard *et al.*, 1973)

Section 2.23.2: Tissue culture media and conditions

DMEM, with sodium pyruvate and glucose (Gibco) : HepG2 (5,10 or 15% FCS), COS 7 (10% FCS) Hepa 1, (10% FCS)

αMEM (Gibco): $C_3H/10T1/2$ (10% FCS)

Glutamine (0.2 mM), foetal calf serum (FCS, at the amount stated above [v/v]), penicillin (15 U/ml) and streptomycin (5 mg/ml) were added to all media. In some cases insulin (Bovine, Sigma) was added to the medium to a final concentration of 1×10^{-7} M.

All cell culture was carried out at 37 °C in CO_2 incubators and manipulations performed in Class 2 Biological safety cabinets; all solutions were pre-warmed at 37 °C prior to addition to the cells unless otherwise stated. Cells were only grown in the medium shown above and so the term "medium" refers to the medium specific to a particular cell line. All cell lines used in this study grow as monolayers in culture.

Section 2.23.3: Freezing and retrieving cell lines

On reaching confluency the cells were detached from the culture flask by addition of 0.1% trypsin, 0.001% EDTA; the solution was incubated until the cell monolayer was seen to detach using light microscopy. The trypsin was diluted following addition of medium and the cells harvested at 3,000 g for 5 minutes. The cell pellet was resuspended at a density of 5-10 million cells/ml in 90% new-born calf serum, 10% DMSO and frozen overnight at -70 °C before transfer to liquid nitrogen for long-term storage. To retrieve cell aliquots from liquid nitrogen the 1 ml sample was quickly thawed at 37 °C and resuspended in medium; the cells were collected following centrifugation at 3,000 g for 5 minutes to ensure removal of most of the DMSO, resuspended in medium and seeded into 25 cm^2 flasks. The cells were allowed to adhere overnight and were re-fed the following morning, by replacement of the medium, to remove any dead cells.

Section 2.23.4: Feeding and sub-culturing of cells

Cells were fed every second day to maintain correct growth conditions. On reaching confluency the cells were washed twice in PBS (Section 2.12.5) and detached from the culture dish and collected as described above (Section 2.23.3). The cell density was estimated using a Neubayer haemocytometer manually under light microscopy. Cells were re-seeded onto fresh plates at a dilution of 1: 10. The viability of cells was estimated by the proportion of cells seen to exclude Nigrosin (Sigma); Nigrosin was prepared as a stock at 0.5% (w/v) in water and added 1: 1 (v/v) to the cell suspension and the level of viable cells was estimated immediately using light microscopy and a Neubayer haemocytometer (Kaltenbach *et al.*, 1958).

Section 2.23.5: Transfection of cell lines with plasmid DNA

For both heterologous protein accumulation studies and promoter assays DNA was transfected into cell lines using the calcium phosphate method similar to that of Graham and van der Eb (1974); the following description is for transfections of 1 X 10^6 cells and larger transfection experiments were scaled up accordingly. Cells were harvested by trypsinisation (Section 2.23.3) and 1 X 10^6 cells were replated in 25 cm^2 flasks and allowed to re-adhere to the flask in 5 ml of medium for 24 hours. 20 µg of the DNA sample to be transfected was mixed with TE/10 (0.1 mM EDTA, 1.0 mM Tris.HCl [pH 8.0]) to a final volume of 225 µl, to this mixture was added 25 µl of 2.5 M $CaCl_2.6H_20$. The DNA/TE/10 and $CaCl_2$ mixture was added slowly, with swirling, to 250 µl of 2 X HBS (0.28 M NaCl, 50 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid), 1.5 mM $Na_2HPO_4.2H_2O$, [pH 7.1]) and left for 30 minutes during which time a fine white precipitate forms. The mixture containing the precipitate was added to the medium bathing the cells and incubated overnight; the following day the DNA containing medium was removed, the cells re-fed fresh medium and the culture allowed to grow for the required time.

Section 2.23.6: Isolation of protein from COS 7 cells

COS 7 cells, transfected with the pCMV4 expression vector (Section 2.23.5), were left for the stated period until harvesting by trypsinisation (Section 2.23.3). The cell density was estimated haemocytometrically (Section 2.23.4) and the cell pellet was resuspended in 125 µl of ice cold buffer H (10 mM Na_2HPO_4, 2 mM $MgCl_2$, 2 mM dithiothrietol, 1 mM EDTA, [pH 7.4]) per 25 cm^2 flask. The cells were sonicated at low power (Soniprep 150 sonicator, MSE) for 2 X 5 seconds and stored on ice between

and after sonication periods. Cellular disruption was confirmed morphologically using light microscopy and the protein concentration of the sample estimated (Section 2.20).

Section 2.23.7: Chloramphenicol acetyl-transferase (CAT) promoter activity assays

Chloramphenicol acetyl-transferase (CAT) promoter activity assays were carried out using a modification of Gorman *et al.* (1982). Cells were plated on 90 mm circular dishes, transfected with the DNA construct and 48 hours after DNA transfection the cells were washed in Mg^{2+} and Ca^{2+} free PBS (per litre: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, [pH 7.4]), detached from the dish using a cell scraper and collected in 1 ml of Mg^{2+} and Ca^{2+} free PBS. Cells were washed by two consecutive 15 seconds periods of centrifugation at 12,000 g and resuspension in Mg^{2+} and Ca^{2+} free PBS. Following this the Mg^{2+} and Ca^{2+} free PBS was aspirated and the cells resuspended in 100 µl of Tris.HCl (pH 7.8). The cells were disrupted by three cycles of freezing in a dry-ice ethanol bath and thawing at 37 °C; the disrupted cellular debris was collected by centrifugation at 12,000 g for 5 minutes and the supernatant containing the CAT was decanted. The level of de-acetylase activity in the supernatant was reduced by heating the samples at 65 °C for 10 minutes. The protein concentration in the supernatant was estimated (Section 2.20) and 50 µl aliquots of equal protein concentration (dependent on the amount of protein present in the lowest protein concentration sample) of all the samples to be assayed were added to 80 µl of CAT reaction mix (50µl 1 M Tris.HCl (pH 7.8), 10 µl of ^{14}C-labelled chloramphenicol diluted to 0.1 mCi/ml, 20 µl of freshly prepared acetyl CoA [3.5 mg/ml in water]). The samples were mixed and incubated for 2 hours at 37 °C. Following the incubation 1 ml of ethylacetate was added to each sample and mixed by vortexing, the phases were separated by centrifugation at 12,000 g for 5 minutes; the acetylated forms of chloramphenicol partition into the upper organic phase and 900 µl of this phase was removed. Ethylacetate was removed from the sample by centrifugation under vacuum for 30 minutes (SpeedVac, Sorvall). The CAT reaction products were resuspended in 25 µl of ethylacetate and 15 µl of this was added to the origin of a thin layer chromatography (TLC) plate in 5 µl aliquots; the site of addition was dried using a hair-drier between applications. The acetylation products were separated in chloroform/methanol (95: 5 [v/v]) in a chromatography chamber; the plate was air dried and sprayed with Enhance (DuPont) to decrease the absorption of the ^{14}C signal by the TLC plate during exposure to X-ray film (Section 2.13).

Section 2.23.8: Transfection of Embryonic Stem (ES) cells by electroporation, positive -negative selection and isolation of genomic DNA

Embryonic Stem (ES) cells were grown in DMEM/Hams F12 media (1:1) on 1% gelatin treated culture flasks in the presence of 100 U/ml leukemia inhibitory factor (LIF). LIF is a glycoprotein which reversibly inhibits the differentiation of the cells (Smith *et al.*, 1988). The cells were detached by trypsinisation (Section 2.23.3), 1 x 10^7 cells were mixed with 100 µg of linearised vector in a final volume of 1 ml PBS (Section 2.16.5) and transfected by electroporation using 0.3 µF capacitance, 0.8 kV in a BRL electroporator. Following electroporation the cells were plated onto 10 x 25 cm^2 plates in 5 ml of media per flask. The following day positive G418 (Geneticin, 200 µg/ml) selection was applied and when resistant clones became visible gancyclovir negative selection was applied (200 µg/ml) for five days. Clones surviving both these selections were picked and grown in 2 x 2 ml culture wells. The cells from one well were frozen (Section 2.23.3) and genomic DNA was prepared from the cells in the second well. Cells were washed in PBS and trypsinised (Section 2.23.3) and disrupted in lysis buffer (500 µl PBS, 5 µl 1% (w/v) SDS, 25 µl proteinase K, from a 10 mg/ml stock) and genomic DNA precipitated following addition of 0.1 volumes of 10 M ammonium acetate and 2.5 volumes of isopropanol. Genomic DNA was spooled from the sample using sealed glass pipettes, eluted into TE (pH 8.0) and DNA concentrations estimated (Section 2.3.6). Maintainance and manipulation of ES cells was performed in collaboration with Dr J. Dorin, MRC Human Genetics Unit, Western General Hospital, Edinburgh.

Chapter 3: The regulation of the CYP2E subfamily, and other xenobiotic metabolising enzymes, by the diabetic state

Section 3.1 Introduction and aims: Physiological and Pathological control of the xenobiotic metabolising and conjugating system

Although it is clear that many chemicals have the capacity to induce xenobiotic metabolism by increasing the levels of certain cytochrome P-450 and other components of the xenobiotic metabolising system the physiological situations in which such changes might occur are less clear. It could be argued that certain components of the cytochrome P-450 superfamily have either evolved solely to function as xenobiotic metabolisers capable of being induced in an organism by an adaptive response to chemical insult, or they have an overlapping endogenous role which is coincidentally perturbed by exogenous chemical challenge. Both physiological and pathological conditions have been seen to modify the activity of "xenobiotic metabolising" enzymes. Conditions such as starvation (Johansson *et al.*, 1988) and diabetes (Dixon *et al.*, 1961) alter the rate of drug metabolism via endogenously mediated modification in these enzyme systems. Arguably in order to truly understand how a system is regulated, and the rationale behind the mechanism and form which this regulation takes, the real function of the system must be established. Insight gained from such an understanding will allow a logical assessment of overlapping changes in the system introduced by non-physiological stimuli mimicking, in some fashion, the endogenous control mechanism. To examine this argument a study of the effect of the diabetic state on some of the components of the P-450 xenobiotic metabolising system and some other components of the body's conjugatory systems was undertaken in the spontaneously diabetic BB/E rat.

Section 3.1.1: Changes in xenobiotic metabolism in Diabetes

The fact that diabetes produces profound effects on drug metabolising systems has been recognised for many years. Initial experiments on the nature of these changes focussed on the effect of the diabetic state on chemical metabolism, later studies attributed the changes seen to modifications in P-450 protein profiles and finally some of the proteins seen to be involved in the changes were purified.

Early work showed that diabetes altered the length of hexobarbital sleeping time in rats treated with the diabetogenic compound alloxan, suggesting changes in clearance rates of drugs in the diabetic state (Dixon *et al.*, 1961). Increased metabolic activity was noted towards a variety of compounds, including an increase in the hydroxylation of aniline (Kato & Gillette, 1965), aryl-hydrocarbon compounds, and androsterone (Reinke *et al.*,

1978). Changes in the activity of sex-dependent P-450 activities were seen to be depressed in male rats (Warren *et al.*, 1983) and it was also noted that the changes in the drug metabolising systems in diabetes generated changes in the susceptibility of the animal to certain hepatotoxins such as carbon tetrachloride (Hanasono *et al.*, 1975 a and b).

The changes in the profiles of the P-450 protein fraction were studied in chemically induced diabetic animals. An increase in a M_r 52,000 band, the characteritic size of P-450 proteins, was seen on two dimensional gels in alloxan induced diabetic animals. This band was not induced by chemicals, such as 3-methlycholanthrene or phenobarbital, which had previously been seen to induce other P-450 isoforms. The characteristics of this diabetes-associated protein band were seen to correlate with, and it was suggested to be the same as, a band previously seen to be induced by alcohol dehydrogenase inhibitors such as pyrazole (Past & Cook, 1982).

The use of antibodies prepared against purified P-450 proteins were used to follow the changes in particular protein levels in diabetes. The protein levels of the rat male specific testosterone 16 α-hydroxylase, now termed CYP2C11, were shown to be decreased in streptozotocin-induced diabetic rats (Favreau *et al.*, 1987a.). The same treatment induced several other P-450 bands which were purified and analysed. The M_r 52,000 protein, previously noted to be induced in diabetes, was purified and antibodies raised to it confirmed the suggestion that it was the same P-450 as induced by pyrazole. The diabetes induced protein was seen to be responsible for some of the marked increase in aniline hydroxylation noted in diabetes and it was also seen to metabolise acetol (Favreau *et al.*, 1987a). the N-terminal sequence, although unique at the time, now places this protein in the CYP2E subfamily. In a similar manner a predominantly male P-450, now placed in the CYP2C7 subfamily, was purified following diabetes-induced elevation. Studies of two-dimensional gels however revealed that these three P-450s were not the only proteins within the P-450 M_r band which were modulated by the diabetic state (Favreau *et al.*, 1987b.) In a similar study, based on the purification of P-450 enzymes seen to be induced by diabetes, CYP2E and another previously uncharacterised enzyme were purified, the substrate profiles analysed and N-terminal sequenced (Imoaka *et al.*, 1988). The second enzyme displayed catayltic activity towards the metabloism of lauric acid, hydroxylating it at the ω an ω-1 positions, activities suggested by its N-terminal sequence similarity to a P-450 previously purified from rat kidney (Imoaka & Funae, 1986). By comparison of the N-terminal sequence of theis enzyme with genomically derived sequences this protein can now placed in the CYP4A, specifically CYP4A2, subfamily.

In summary initial biochemical studies, followed by purification and immunologically based investigations, revealed that the levels of several forms of P-450 were both elevated and depressed in diabetes. Several lines of investigation began to converge as it was seen that chemicals, such as alcohol dehydrogenase inhibitors, could produce changes in certain

P-450 isoforms similar to those seen in diabetes suggesting an overlap in the physiological and xenobiotic control mechanisms operating on this enzyme system.

Section 3.2. Diabetes mellitus

Diabetes mellitus is a common pathological state affecting 2-4% of the population in industrialised countries. The two commonest forms of the disorder are "Type I, insulin-dependent diabetes mellitus" (IDDM), and "Type 2, non-insulin dependent". Type 2 diabetes generally occurs in older, often overweight, people and has a progressive onset often associated with dysfunction of insulin secreting cells and peripheral resistance to insulin; Type 2 diabetes will not be discussed further. Type I diabetes is caused by the destruction of the insulin secreting β-cells within the pancreatic Islets of Langerhans. The disorder is insidious in onset with a slow destruction of the β-cells by auto-immune processes, leading to "insulitis" (infiltration of the pancreas by mononucleur cells) over a period of several years (Tarn *et al.*, 1987). A genetic link between the incidence of the disorder and the major histocompatibility complex gene locus has been established (Todd *et al.*, 1987) and it is clear that environmental factors also play a large part in the development of the disorder. The only available treatment for the disorder is insulin replacement therapy which, inspite of now having been available for more than sixty years, still does not remove some of its debilitating effects. As a consequence diabetic subjects have a significant reduction in life-expectancy, around 30%, generally attributable to the effects of inadequate metabolic control afforded by insulin replacement therapy (Green & Hougard, 1984). Excess mortality is largely accounted for by vascular disease and hypertension, although other complications, akin to an acceleration of aging, are also seen (Krowleski *et al.*, 1988). The lack of metabolic control in diabetic subjects leads to higher fluctuations in blood sugar levels than in non-diabetic sujects. Blood sugar can react with proteins by a condensation reaction between the sugar aldehyde group and the protein amino group in a series of events termed the "Maillard" or "browning" reaction. This reaction generates a stable ketoamine, or "Amadori" product on proteins with both a long, for example collagen and myelin, and short half-life, for example haemoglobin, in the body. The generation of these products leads to the detectable presence of glycated haemoglobin, termed HbA1, in the circulation of diabetic subjects which is often used as a means of ascertaining an individuals level of metabolic control as it correlates with the ambiant blood glucose levels (Cerami, 1988). In long half-life proteins Amadori products can further react with each other, via dehydration reactions possibly involving radical initiated reactions, generating cross-linking bridges within or between molecules, termed "advanced glycosylation end" (AGE) products. These processes occur in all individuals, for example the concentration of Amadori and AGE products in the skin collagen is correlated with age, but, due to the

higher fluctuations a diabetic subjects blood glucose level, these processes are accelerated in diabetes (Dominiczak *et al.*, 1990). The acceleration of these processes is thought to account for many of the complications of diabetes including hypertension, via arterial and cardiac stiffening, nephropathy associated with the thickening of the glomerular basement membrane, collagen cross linking leading to joint stiffening, myelin glycation and neuropathy, and cataract development (Dominiczak, 1991).

Section 3.2.1: Animal models of Type I diabetes mellitus

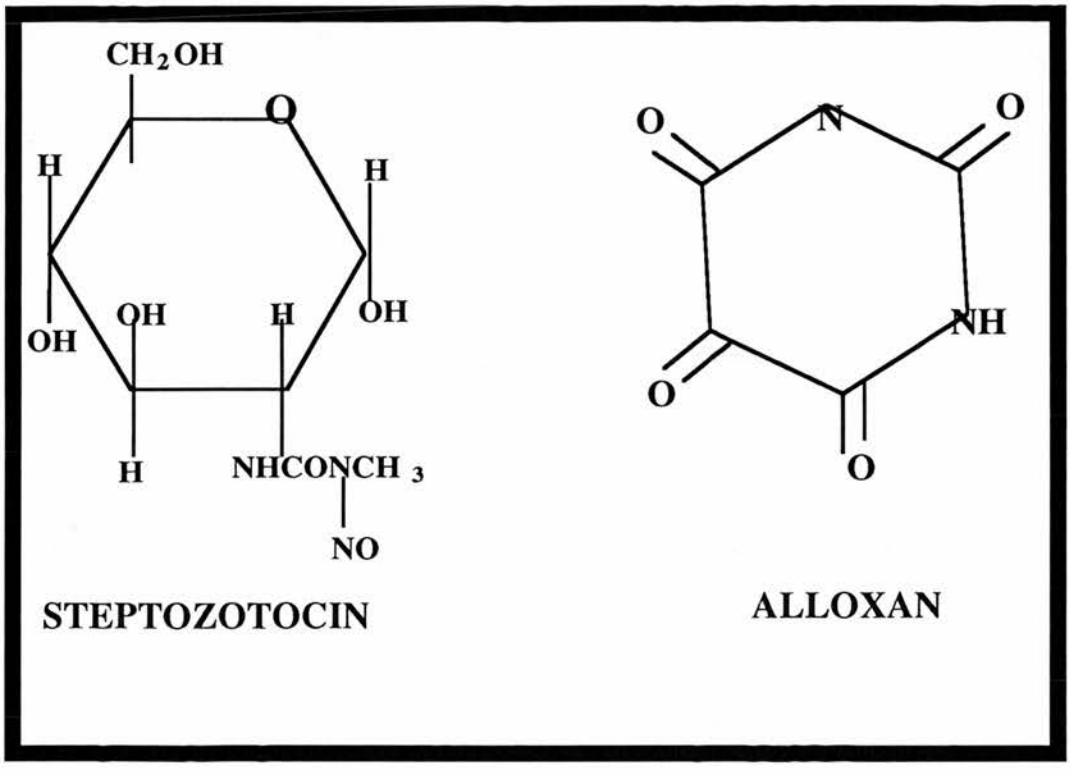
a) Chemically induced diabetes

Most studies into the effects of diabetes on the enzymes of the drug metabolising systems have used chemically induced animal models. The two diabetogenic chemicals most commonly used in these studies are detailed below with the possible problems associated with such an interventionist approach for the generation of the disorder are discussed; the structures for these chemicals is given in Figure 3.1.

Alloxan: As is common for the whole group of diabetogenic chemicals alloxan is thought to act as a diabetogenic agent through an exploitation of the very high susceptibility of the β-cells to damage generated by free-radicals. Within the β-cell alloxan has been seen to react with sulphydryl-protein residues and alters the membrane permeability. Free-radicals produced during oxidation and reduction cycling of alloxan produce protein and DNA damage and increase the rate of DNA repair (Yamamoto *et al.*, 1981).

Streptozotocin: As with alloxan the diabetogenic property of streptozotocin appears to relate to its ability to generate free-radicals within the β-cells.This is emphasised by the observation that diabetogenic action of streptozotocin can be prevented by induction of free-radical scavenging systems such as super-oxide dismutase. Insulitis is seen following treatment with streptozotocin suggesting that the damage produced by the chemical may cause antigen release from the β-cell leading to an auto-immune response accelerating the destruction of the β-cells initiated by radical damage (Robbins *et al.*, 1980; Yamamoto *et al.*, 1981).

Figure 3.1: The structures of the two chemicals most commonly used to generate diabetes in experimental animals.



Chemically induced animal models of diabetes: drawbacks in terms of their utility in the investigation of xenobiotic metabolising systems

Despite the use of such diabetogenic chemicals to produce a diabetes-like state for the study of changes in xenobiotic metabolising enzymes, several problems prevail in such systems. The destructive effects of the diabetogenic chemicals are not directed solely at the β-cells and so extra-pancreatic toxicity of the chemical, and associated shock, cannot be ruled out in the interpretation of results obtained by these methods. It was noted for example that in using streptozotocin to generate diabetes in mice profound changes in the ultrastructure of the hepatocytes were also produced. Degranulation of the endoplasmic-reticulum, mitochondrial swelling, and dilation of the smooth endoplasmic-reticulum, were all seen in the hepatocyte as a result of the administration of streptozotocin. These effects were noted in the hepatocytes after a single injection of streptozotocin whether the animal displayed diabetic symptoms or not (Languens*et al.*, 1980). Studies employing chemically induced diabetes have an added layer of complexity in that a direct effect of the chemical, which generates the diabetic state, may also be producing other diffuse pathological changes perturbing the levels of xenobiotic metabolising enzymes directly. A clear example of this

was seen in the different effects on the activity of *N*-acetylases produced by diabetes in spontaneously, and chemically-induced diabetes in rats. In streptozotocin-induced diabetic animals the rate of acetylation of drugs such as sulphamethazine was seen to be markedly decreased in the diabetic animals (Lindsay & Baty, 1989). By contrast, in the spontaneously diabetic BB rats the rate of acetylation was increased with onset of diabetes (Lindsay & Baird, 1990).

b) A spontaneously diabetic animal model for diabetes mellitus: the BB rat

The BB rat was first identified in a commercial breeding colony, Bio-Breeding Laboratories hence "BB", in 1974 (Chapell & Chapell, 1983). The animal presents an excellent model for type I diabetes displaying many of the metabolic, physiological and functional changes associated with the human disorder (Baird, 1989). Once diabetic animals undergo structural changes in the retina, glomerular basement membrane, and nerves and display clinical characteristics, such as weight loss despite hyperphagia, polyuria, and ketoacidosis, leading to death if insulin is withheld. Diabetes in the animal also generates metabolic changes such as hypoinsulinaemia, post-prandial hyperglycaemia, hyperketoneamia, hyperlipidaemia, and proteinuraemia reflecting muscle protein catabolism (Tannenbaum *et al.*, 1981a; Marliss *et al.*, 1983). The metabolic disorder in the BB rat, as in the human, appears to be auto-immune in nature, with the presence of circulating antibodies against insulin and islet cell-surface antigens (Dean *et al.*, 1987). A peak in the incidence of diabetes occurs when the rats reach sexual maturity, between 60 to 120 days after birth. As has been observed with the human MHC locus, an association with the disorder and the nature of the rat MHC equivalent, the RT1 locus, has been seen (Butler *et al.*, 1983). The destruction of the β-cells, associated with the insulitis following the onset of diabetes in the rats, is highly specific and the glucagon and other hormone secreting cells within the pancreas are left intact, and insulitis disappears after the β-cells are destroyed (In't Veld & Pipeleers, 1988). The mechanism of β–cell destruction is unclear but the fact that β-cells are highly susceptible to free-radical damage, as noted in the proposed mechanism of action of diabetogenic chemicals, suggests that it may be mediated by radicals. Addition of antioxidants in the diet, such as vitamin E, protect the β-cells from destruction suggesting further that free radical damage is responsible for their removal (Malaisse, 1982; Assayam *et al.*, 1986. Nomikos *et al.*, 1986).

As seen in human IDDM studies, where around 50% of monozygotic twins are discordant for the development of IDDM (Leslie & Pyke, 1980), only around 50-80% of animals in a single litter produced by a diabetes-prone (DP) BB rat develop the disorder. This suggests a clear environmental role involving fators such as diet, stress or infection, in the development of diabetes. Theuse of the seemingly non-diabetic, but diabetes-prone

animals, as controls against which the changes observed in the diabetic litter-mates can be compared is innapropriate. The seemingly non-diabetic animals can still display mild metabolic disturbances without having lost enough of their β-cells to need insulin administration to survive. The use of the diabetes-prone animals not developing diabetes to establish, by long term breeding, a diabetes-resistant subline (DR), which displays an incidence of diabetes of less than 1%, however circumvents this problem and these animals serve as an effective control without the introduction of any strain related differences.

Section 3.3: Hormonal and metabolic changes produced by the diabetic state

It is possible that the modifications seen in cytochrome P-450 levels at the onset of diabetes relate to the hormonal and metabolic changes ensuing from the disorder. The major changes in hormonal levels and their metabolic effects in the diabetic state are outlined below. The changes in some, or all of these parameters may represent components of the endogenous signaling systems controlling the xenobiotic metabolising enzymes of the P-450 system. Understanding the role of these changes in the modulation of these systems may allow an appreciation of the mechanisms whereby some components of the P-450 drug metabolising system are physiologically controlled.

Section 3.3.1: Metabolic changes in diabetes

The loss of insulin, resulting from of the destruction of the β–cells in diabetes, prevents most cells taking up, storing, and using glucose as a metabolic fuel source. As a result of insulin loss post-prandial circulating levels of glucose are high, the renal glucose threshold is passed and glucose is excreted in the urine. The body attempts to compensate for the seeming lack of glucose by increased release of glucagon, simplistically the antagonist to insulin, and the actions of this hormone lead to increased levels of gluconeogenesis, glycogenolysis, lipolysis and a switching to the use of fatty acids as the main metabolic fuel supply. With the prolonged use of free-fatty acids as a fuel source circulating levels of ketone bodies rise. The processes of attempted glucose homeostasis in diabetic subjects are essentially exaggerated and ineffectual versions of those employed in the body of the non-diabetic subject during moderate fasting, for example overnight, or more prolonged starvation. The main control of the fuel source used by the body is thought to be dictated by the fuel used by the muscles. In a fasted, starved or diabetic state the insulin:glucagon ratio falls and the circulating levels of blood lipid, either through increased lipolysis or decreased re-esterification of free-fatty acids, rise. The increase in blood free-fatty acid levels leads to

a decrease in use of glucose in the muscle as citrate, produced by β-oxidation, inhibits glycolysis by reducing the activity of phosphofructokinase, and the uptake of glucose.
The switch from the use of glucose to the use of free-fatty acids by the muscles means that other tissues more dependent on glucose, such as the intestine, brain and kidneys, can use the remaining glucose as the ambient levels fall. Increased levels of gluconeogenesis are induced by changes in the insulin:glucagon ratio producing phosphorylation changes within cells (Section 4.14.1). Glycerol, lactate and eventually, with prolonged starvation, certain amino-acids produced by the breakdown of muscles, are used to fuel gluconeogenic processes. Following feeding, in the presence of active β–cells, glucose levels rise causing an increase in the insulin: glucagon ratio and the metabolic processes are reversed. The liver takes up and uses glucose, replenishes glycogen stores and the rate of free-fatty acid release is inhibited. As the muscles stop using free-fatty acids as a fuel source, citrate levels fall and the rate of glycolysis rises. In a diabetic state the β-cells are not present to an effective level and so, even in a fed-state, the insulin:glucagon ratio remains low and a pseudo-starved metabolic state is maintained (Johnston *et al.*, 1984; Pilkis *et al.*, 1988).

Section 3.3.2: Ketone body metabolism and ketosis in diabetes

β-oxidation of free-fatty acids generates acetyl-coenzyme A (acetyl CoA) which in a non-starved state feeds into the Krebs cycle generating citrate from oxaloacetate. The flux through the Krebs cycle is fairly constant whereas, with increased levels of circulating free-fatty acids in the fasted, starved or diabetic states, the activity of the mitochondrial β-oxidation pathway varies. When the insulin:glucagon ratio falls, and increased lipolysis occurs, a rise in the level of fatty-acyl coenzyme A entering the mitochondria occurs as the activity of palmitoyl coenzyme-A transferase I, generating the substrate for the mitochondrial carnitine shuttle, increases. As levels of acetyl-CoA produced by the increased β-oxidation build up, and the levels of oxaloacetate fall, acetoacetate is produced from two molecules of acetyl-CoA. Acetoacetate is enzymatically reduced to β-hydroxy butyrate which can be used as a fuel source by peripheral tissues folowing its re-oxidation to acetoacetate and the regeneration of two acetyl-CoA molecules. In the staved or diabetic state, however, the uncontrolled increase in acetoacetate production cannot all be used by peripheral tissues as glycolysis fails. This leads to high levels of circulating acetoacetate and β-hydroxybutyrate as well as, due to decarboxylation of acetoacetate, the generation of acetone. Collectively these processes produce ketosis in diabetes and the effect which this generates on the pH of the blood is of critical importance in the acute damage induced in diabetes (Johnston *et al.*, 1982; Johnston *et al.*, 1982).

Section 3.3.3: Modifications in hormonal status in diabetes

Essentially it is difficult to dissect the metabolic and hormonal changes experienced in the diabetic state. A change in the metabolic status elicits a change in hormonal status leading to the generation of a feed-back loop which, in a non-diabetic subject, generates the most efficient use of the metabolites available. As a result most changes in hormonal status produce a negative feed-back loop controlling the level of hormonal secretion either directly or indirectly; the antagonistic nature of insulin and glucagon reaffirms this hormonal and metabolic interrelationship.

In the diabetic state glucagon levels rise leading to the stimulation of gluconeogenesis, lipolysis, and glycogenolysis, and the levels of glycolysis and glycogen synthesis are repressed; in a non-diabetic individual the secretion of insulin is also elevated. Insulin antagonises the actions of glucagon and also stimulates glucagon secretion. In the diabetic state insulin levels fall as the β-cells are destroyed. As well as the effect of diabetes on these two hormones other circulating hormonal levels become modified and these too play a part in the metabolic changes occuring. The level of somatostatin (somatotrophin-release inhibiting factor, or SRIF) rise; SRIF is a hormone which has an inhibitory effect on the actions of both glucagon and insulin and a variety of gut hormones and growth hormone (Mortimer, *et al.* , 1974). Diabetes also produces profound effects on the circulating levels of growth hormone in some species; studies of the ultradian secretion of growth hormone in male BB rats showed that, with the onset of diabetes, the levels of growth hormone fell. This fall in circulating growth hormone levels was reflected in a decrease in both the peak height and the amplitude of the growth hormone release pulse (Tannenbaum *et al.*, 1981b) and this was also observed in streptozotocin treated rats (Tannenbaum, 1981b). The decrease in growth hormone levels may relate to the effect of the diabetic state on the levels of SRIF, as mentioned the rise in SRIF decreases the level of growth hormone release. Insulin also has been shown to generate transcriptional activation of the growth hormone promoter and loss of this activation in the diabetic state may also account for the drop in the level of growth hormone (Isaacs *et al.*, 1987). Starvation produces a similar drop in the level of circulating growth hormone in the rat, presumably acting through similar mechanisms as those which operate in diabetes (Tannenbaum, 1979). In human diabetic subjects the levels of growth hormone, by contrast, are reported to be elevated (Asplin *et al.*, 1989); similarly growth hormone levels become elevated in starved human subjects potentially complicating the cross-species interpretation of changes which may be caused by modifications of growth hormone levels (Hansen & Johansen, 1970; Ho *et al.*, 1988).

Section 3.3.4: Global changes in the translation and degradation rates of proteins in starvation and diabetes

As well as changes in intermediary metabolism the hormonal changes in diabetes, and starvation, produce a series of changes in the translational and degradational systems within the body. In general these changes generate a non-specific effect, but it is clear that their roles have ramifications in understanding the molecular basis for any modifications in enzyme systems in the starved or diabetic state.

Phosphorylation and dephosphorylation changes, as a result of hormonal actions, generate modified activity in numerous components of the translational machinery leading to changes in the translation rates of transcripts in general. There appears to be no general theme of, for example, increased phosphorylation or dephosphorylation alone generating a decrease or increase in the translation rate, rather a complex combination of both phosphorylation and dephosphorylation of various components affects the ribosomal translational efficiency. The actions of insulin increases the level of phosphorylation on the initiation factors eIF-4B (a helicase), eIF-4F (a cap recognition protein), the ribosomal associated eIF-3 as well as the ribosomal S6 protein in a co-ordinate manner leading to an increase in the rate of translation. Serum starvation leads to loss of phosphorylation at these sites and decreases translational rates (Marino *et al.*, 1989). Thus the overall level of translation in the diabetic state, with loss of phosphorylation of these ribosomal components due to the loss of insulin, would be expected to be depressed.

Starvation leads to an increase in the level of degradation of a variety of proteins by the induction of the level of macro- and microautophagy of proteins and organelles by lysosomes. These pathways appear to be non-selective in the proteins or organelles which they degrade. The elevations in activity of the lysosomal degradative pathways occur predominantly in tissues which atrophy in starvation such as muscle, liver and kidney and these processe may contribute to the provision of gluconeogenic amino-acid substrates (Dice, 1987; Mortimore *et al.*, 1988). In the rat the general activation of autophagasomal pathways are supplemented, following two days of fasting, by the specific increase in a selective lysosomal degradative pathway. The activation of the selective lysosomal pathway leads to an increase in the turnover of a class of proteins, such as aspartate amino transferase and pyruvate kinase, with a common lys. phe. glu. arg. gln motif which presumably indicates to the degradative machinery that these proteins are dispensible to the cell and can be selectively used as gluconeogenic precusors in starvation (Dice *et al.*, 1986).

Thus in the starved and diabetic states a general decrease in the overall levels of message translation, due to a depression in the activity of ribosomes, and an increase in the level of

both non-specific and specific pathways for protein degradation form the background to any changes in the drug metabolising enzyme system components.

Section 3.4: Changes in the drug metabolising enzyme systems of the BB/E (Edinburgh) rat with onset of type I diabetes

Using the spontaneously diabetic BB/E rat changes in cytochrome P-450 enzymes and components of the phase 2 conjugating systems were investigated at a molecular level. Using several antibodies and cDNA probes the changes induced by the diabetic state, and the possibility of their reversibility by insulin, were studied. Both male and female rats were used in this study.

Diabetes-prone (DP) animals were weighed three times a week, and the urine of animals seen not to gain weight was analysed for the presence of glucose. If glucose was seen to be present in the urine then blood glucose analysis was performed. From these results those animals which displayed a blood glucose level of, or higher than, 18 mmoles per litre were classed as diabetic. One group of diabetic DP animals detected in this manner was left for three days without administration of insulin and then sacrificed; in subsequent references to this group, these animals are termed DM (Diabetic male), or DF (diabetic female). A second group of diabetic DP animals were left for the same period after detection of diabetes but then were treated with insulin and their blood glucose levels were returned to normal, around 2-4 mmoles per litre, prior to sacrifice; in subsequent references to this group, these animals are termed IM (insulin treated male), or IF (insulin treated female). Age and sex matched diabetes-resistant animals were taken to act as controls to the possible changes induced by diabetes, and their potential reversal by insulin treatment; these animals are referred to as SM (subline male) and SF (subline female) in subsequent analysis.

Biochemical analysis of blood samples taken from the animals was performed to establish possible links between the metabolic changes seen and modifications in the xenobiotic metabolising systems (Table 3.1). To analyse the potential role played by the elevated levels of ketone bodies in the diabetic state in the production of changes in the P-450 system a separate study was undertaken where blood ketone levels were experimentally elevated. In this study both male and female Wistar rats, the progenitor strain for the BB rat, were given 1% acetone in their drinking water over a two week period. In addition to these animals a third group of animals were studied which underwent the two week acetone treatment, but were then placed back on standard drinking water to study the longevity of acetone induced changes in P-450 levels.

	CHOLESTEROL mmol/l	TRIGLCERIDES mmol/l	F-F. ACIDS mmol/l	β-HYDROXY-BUTYRATE mmol/l	INSULIN mU/l	GHb %	n
SUBLINE MALE	2.42 (0.28)	1.22 (0.31)	0.31 (0.09)	0.209 (0.01)	89.3 (90.0)	3.4 (0.7)	4
SUBLINE FEMALE	2.49 (0.19)	1.09 (0.45)	0.41 (0.13)	0.121 (0.01)	38.1(30.4)	3.3 (0.5)	4
DIABETIC MALE	3.03 (0.82)	4.83 (4.90)	0.98 (0.28)	3.203 (3.30)	nd<2.5	11.4 (1.8)	3
DIABETIC FEMALE	2.52 (0.13)	2.29 (1.87)	0.94 (0.3)	2.661 (1.92)	nd<2.5	7.7 (2.2)	3
INSULIN MALE	3.16	1.25	0.59	0.297	25	6.7	
INSULIN FEMALE	1.70 1.67	0.81 0.70	0.28 0.52	0.117 0.091	1225 500	6.3 7.2	

Table 3.1: Biochemical analysis of components of the blood and plasma in the BB/E rats used in this study. Individual components of the blood and plasma taken from the BB/E rats used in this study were analysed to assess the levels of changes generated by the diabetic state and their reversal following insulin treatment. Abbreviations: **F-F. ACIDS:** Free fatty acids; **GHb:** Gylcated haemoglobin. The level of glycated haemoglobin is used as an index of the ambient levels of glucose present in the blood. This figure, rather than a direct measurement of the glucose level, gives a clearer idea of the ambient glucose levels ; the actual glucose levels, even in non-diabetic rats, fluctuate greatly due to the feeding habits of the animal. Great inter-individual variations in the levels of triglycerides and β-hydroxybutyrate components were noted, particularly in the diabetic male animals. For example β-hydroxybutyrate levels of 7.002, 1.968, and 0.639 mmoles per litre, and free fatty acids from the same diabetic male animals of 10.37, 3.30, and 0.82 mmoles per litre were recorded. These results suggest that there is a great inter-individual variation in the severity of the disorder in the diabetic animals. Analysis was carried out in collaboration with Dr R. M. Lindsay, The Metabolic Unit, University of Edinburgh Department of Medicine, Western General Hospital, Edinburgh.

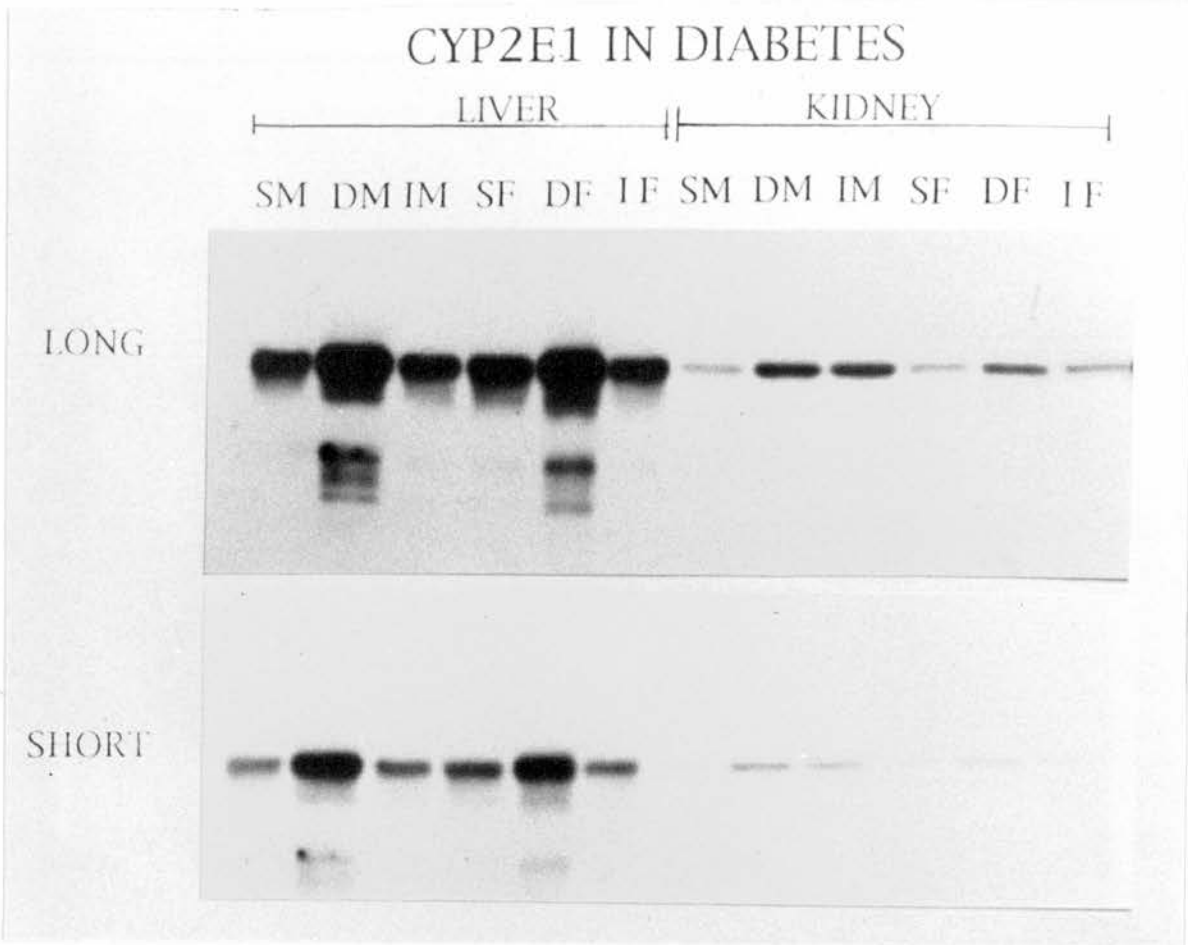


Figure 3.2: Western blot analysis of the effect of diabetes on the protein levels of the CYP2E subfamily in the BB/E rat. Microsomal protein (10μg of liver and 20μg of kidney) isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals were separated by SDS/PAGE on 9% gels.The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies raised to purified rat CYP2E1 protein. No bands other than those shown were detected. The CYP2E protein ran with a M_r of approximately 52,000. The long (**LONG**) and short (**SHORT**) exposures of the blot allow a better assessment of the induction levels of CYP2E in the two tissues.

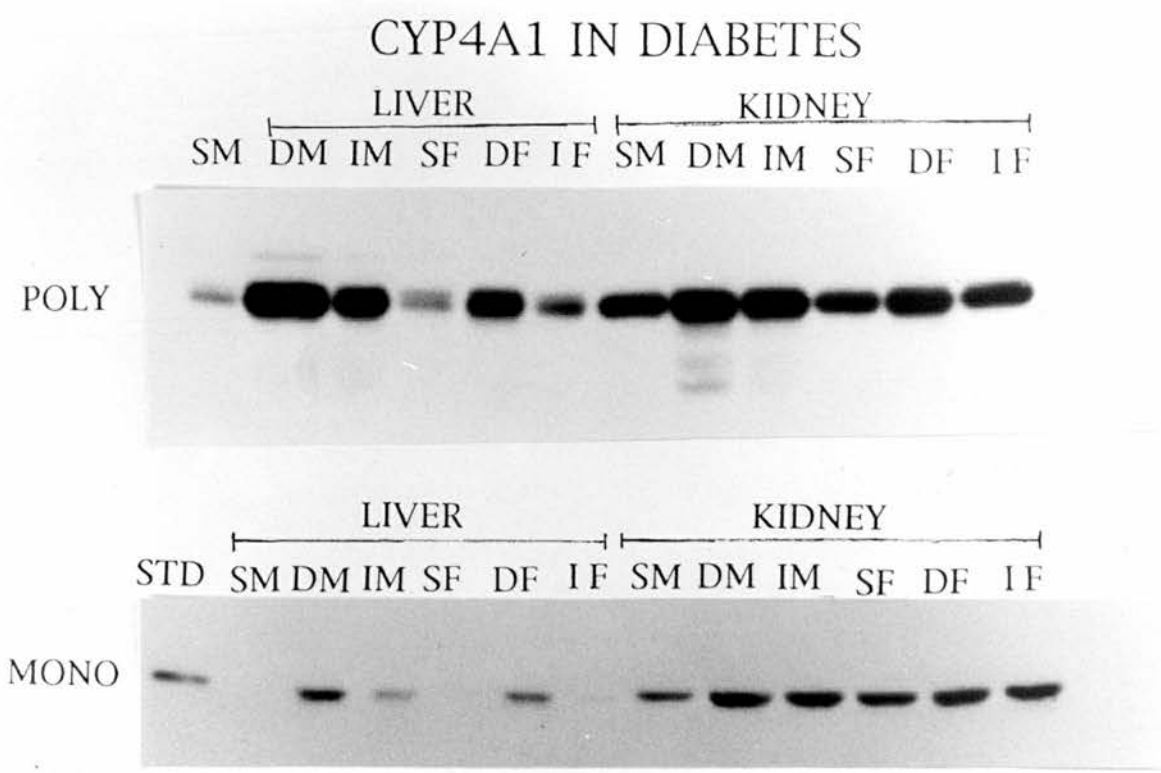


Figure 3.3: Western blot analysis of the effect of diabetes on the protein levels of the CYP4A subfamily in the BB/E rat. Microsomal protein (10μg of liver and 20μg of kidney) isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals were separated by SDS/PAGE on 9% gels. The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies (**POLY**) raised to purified rat CYP4A subfamily proteins and a monoclonal antibody (**MONO**) raised to purified rat CYP4A1 protein. No bands other than those shown were detected and the P-450 subfamily proteins ran with a M_r of approximately 52,000. The CYP4A1 standard (**STD**) was isolated from rat liver.

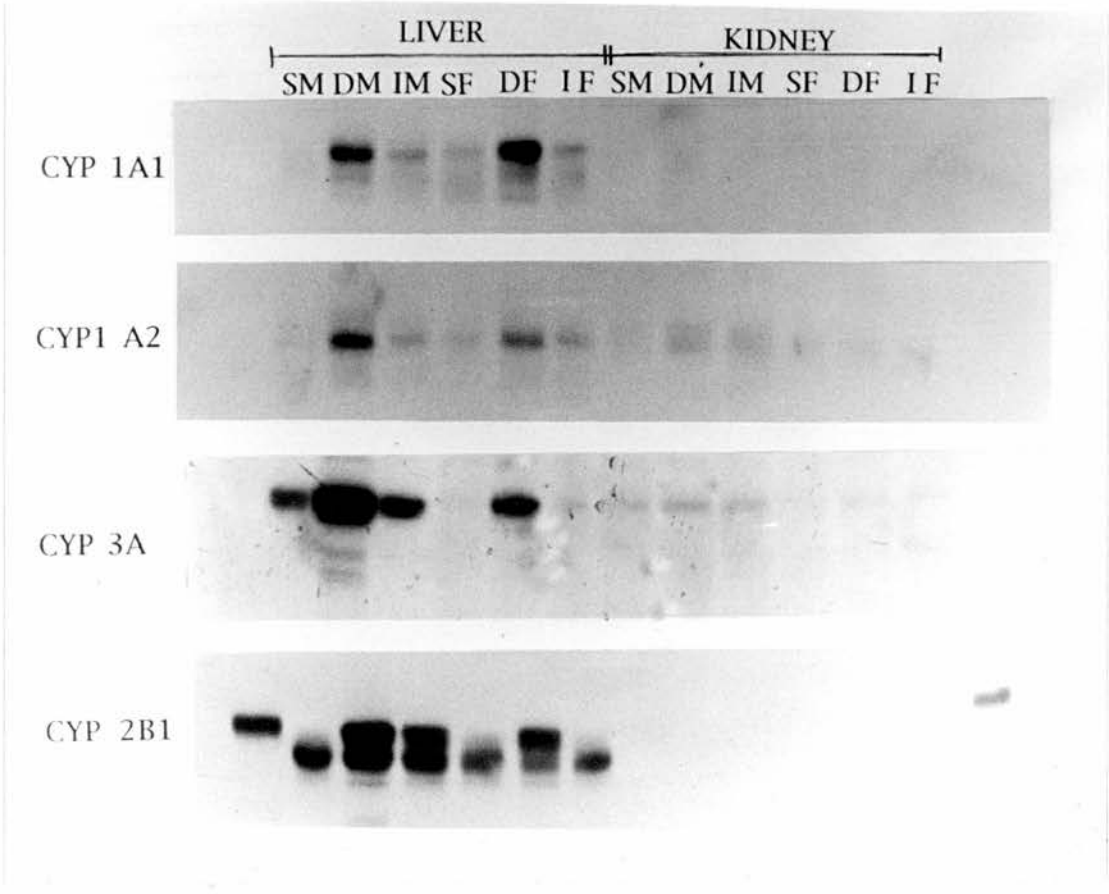


Figure 3.4: Western blot analysis of the effect of diabetes on the protein levels of the CYP1A, CYP2B and CYP3A subfamilies in the BB/E rat. Microsomal protein (10μg of liver and 20μg of kidney) isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals were separated by SDS/PAGE on 9% gels. The proteins were transferred to nitrocellulose filters and probed with polyclonal antibodies raised to purified rat P-450 subfamily proteins as indicated; no bands other than those shown were detected. The P-450 protein bands all ran with a M_r of approximately 52,000. The CYP2B1 standard marks the upper cross-reacting band as CYP2B1 and was isolated from rat liver.

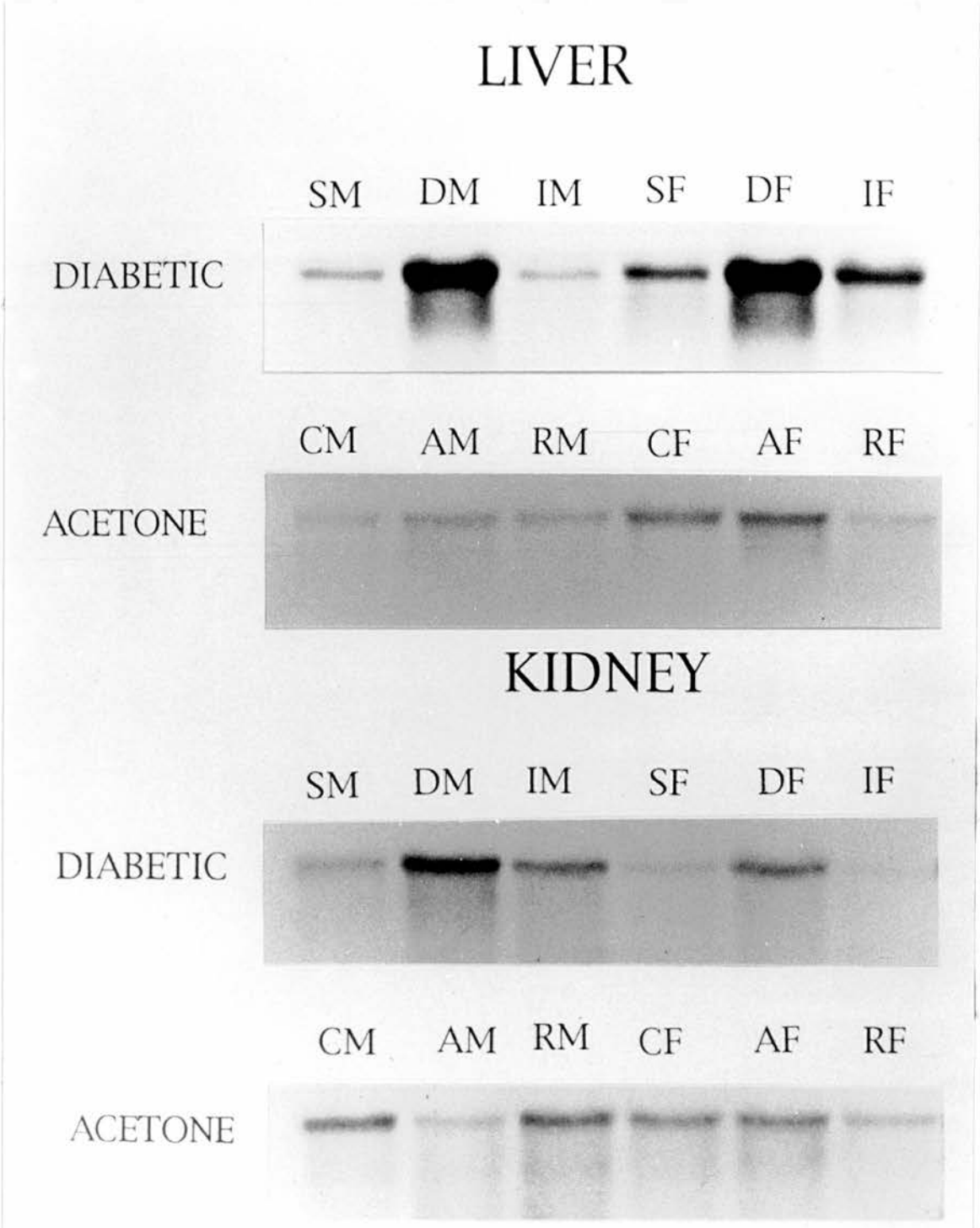


Figure 3.5: The effect of diabetes and acetone treatment on the CYP2E1 mRNA level in the BB/E rat and Wistar rat. Total RNA was isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals and from Wistar control male and female (**CM** and **CF**), male and female acetone treated by 1% acetone addition to drinking water (**AM** and **AF**) and animals allowed to recover from this treatment subsequently by one week with standard drinking water (**RM** and **RF**). 10μg of each RNA sample was subjected to horizontal gel-electrophoreses and transferred to nylon filters by capillary action. The filters were hybridised with a radioactively labelled probe generated from an 800bp mouse partial Cyp2e1 cDNA. No bands other than those shown were detected and the CYP2E mRNA ran with a size of approximately 1,800 bases.

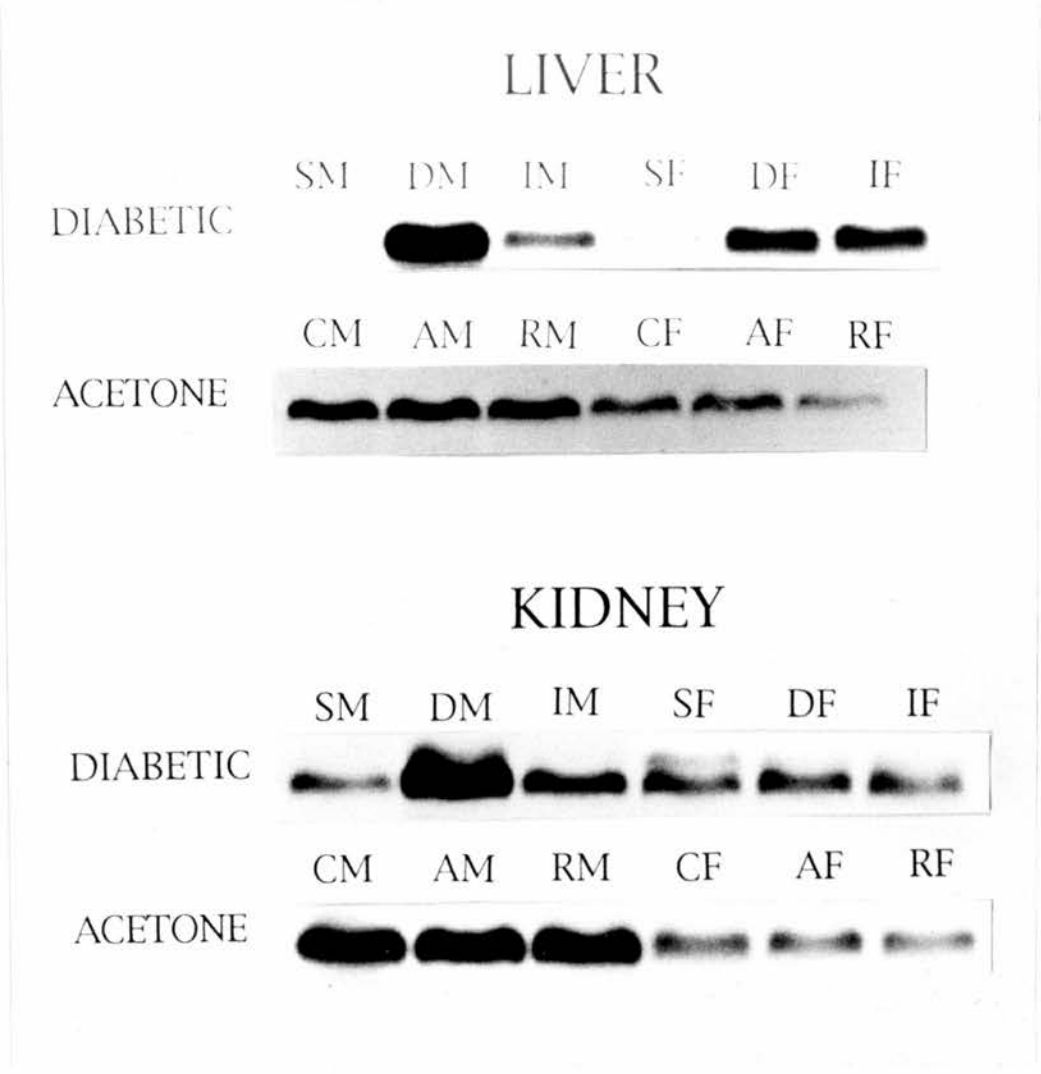


Figure 3.6: The effect of diabetes and acetone treatment on the CYP4A1 mRNA level in the BB/E rat and Wistar rat. Total RNA was isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals and from Wistar control male and female (**CM** and **CF**), male and female acetone treated by 1% acetone addition to drinking water (**AM** and **AF**) and animals allowed to recover from this treatment subsequently by one week with standard drinking water (**RM** and **RF**). 10μg of each RNA sample was subjected to horizontal gel-electrophoreses and transferred to nylon filters by capillary action. The filters were hybridised with a radioactively labelled probe generated from a rat CYP4A1 cDNA. No bands other than those shown were detected and the CYP4A1 mRNA ran with a marker size 2,300 bases.

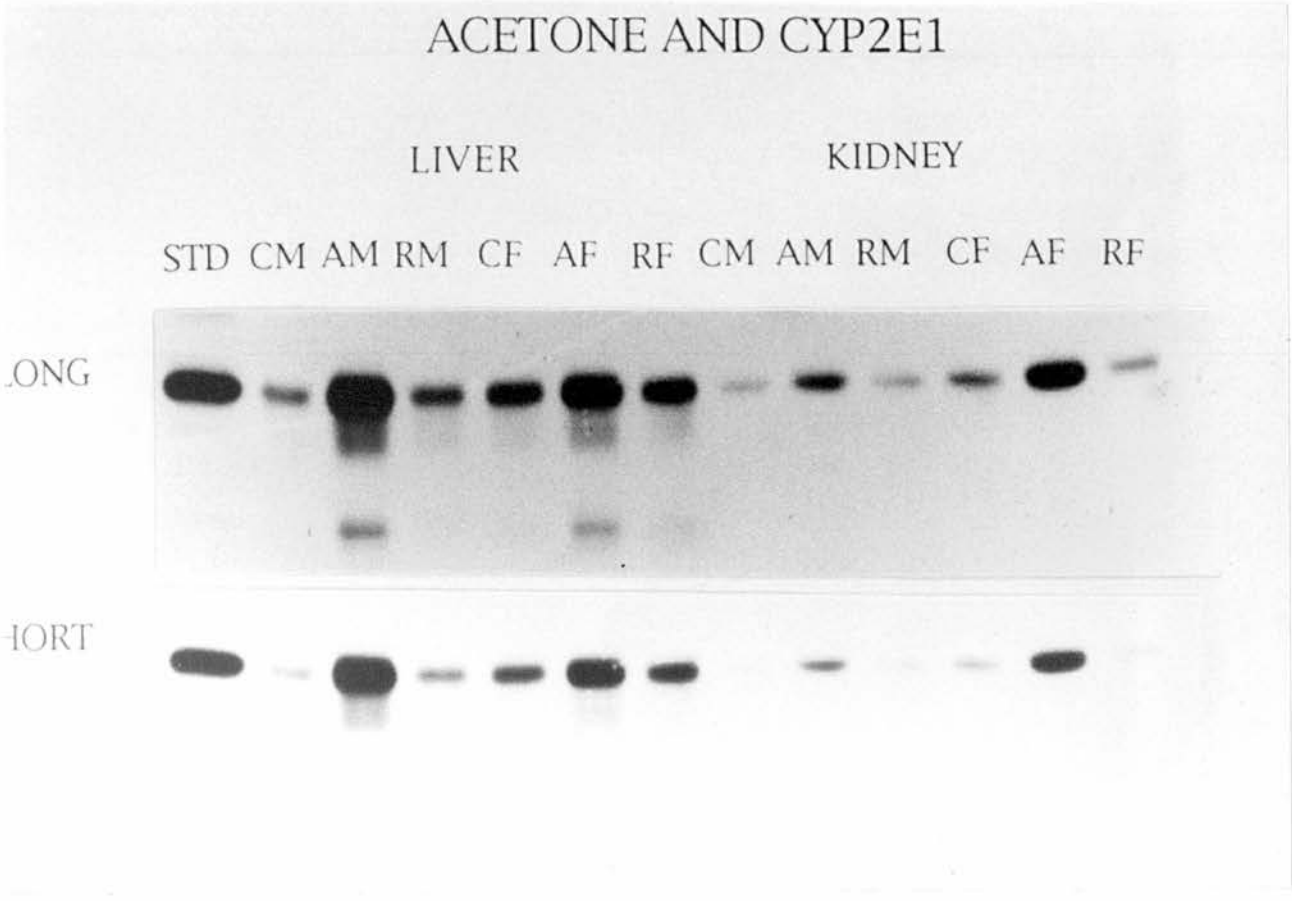


Figure 3.7: Western blot analysis of the effect of acetone treatment on the protein levels of the CYP2E subfamily in the Wistar rat. Microsomal protein (10μg of liver and 20μg of kidney) isolated from control male and female (**CM** and **CF**), male and female acetone treated by 1% acetone addition to drinking water (**AM** and **AF**) and animals allowed to recover from this treatment subsequently by one week with standard drinking water (**RM** and **RF**) were separated by SDS/PAGE on 9% gels. The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies raised to purified rat CY2E1 protein. No bands other than those shown were detected and the CYP2E protein ran with a M_r of approximately 52,000. The CYP2E1 standard (**STD**) was prepared from the membranes of *E. coli* heterologously expressing the mouse Cyp2e1 protein. The long (**LONG**) and short (**SHORT**) exposures of the blot allow a better assessment of the induction levels of CYP2E in the two tissues.

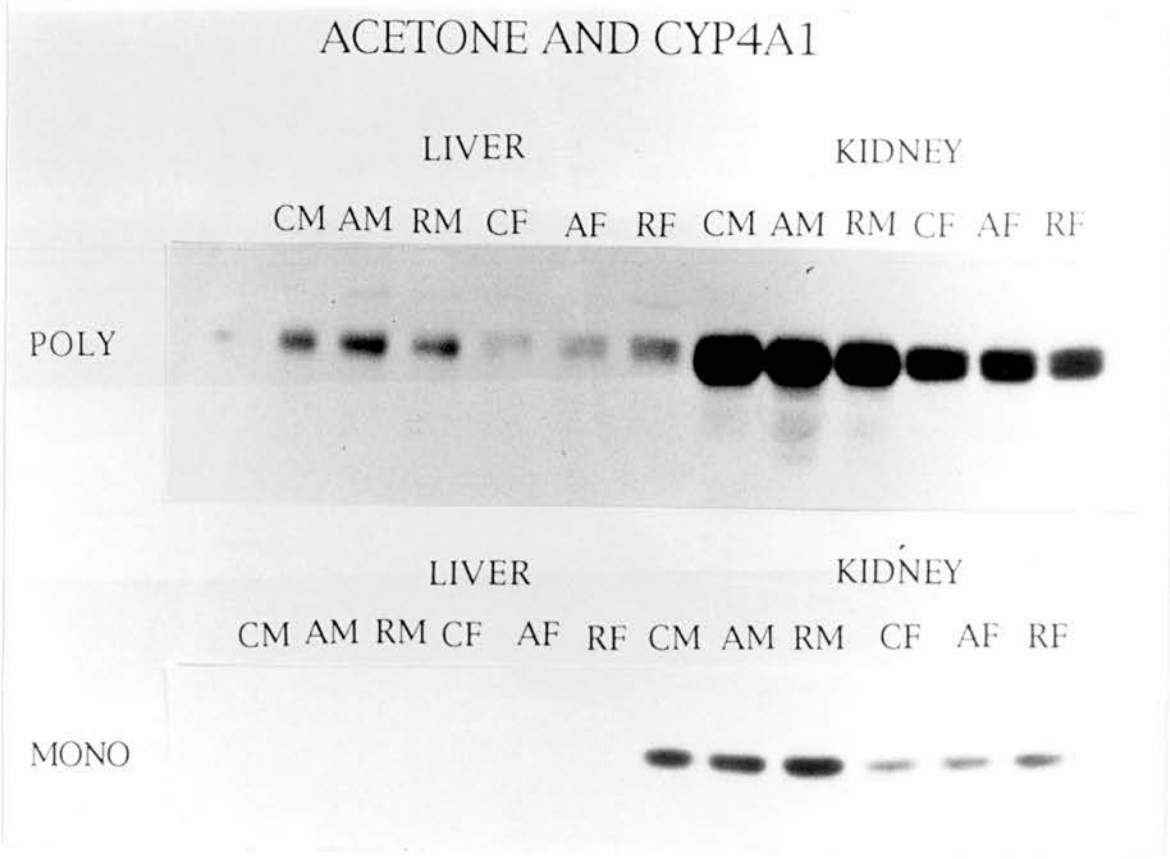


Figure 3.8: Western blot analysis of the effect of acetone treatment on the protein levels of the CYP4A subfamily in the Wistar rat. Microsomal protein (10μg of liver and 20μg of kidney) isolated from control male and female (**CM** and **CF**), male and female acetone treated by 1% acetone addition to drinking water (**AM** and **AF**) and animals allowed to recover from this treatment subsequently by one week with standard drinking water (**RM** and **RF**) were separated by SDS/PAGE on 9% gels. The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies (**POLY**) raised against purified rat CYP4A subfamily proteins and a monoclonal antibody (**MONO**) raised against purified rat CYP4A1 protein. No bands other than those shown were detected. The P-450 proteins ran with a M_r of approximately 52,000.

Section 3.4.1: The effect of the diabetic state on the protein level of a variety of xenobiotic metabolising P-450

The diabetic state leads to a marked induction of the protein levels of all the subfamilies studied. The protein levels of the CYP2E subfamily (Figure 3.2) , CYP4A subfamily, in which the polyclonal antibody employed has previously been seen to recognise epitopes of both CYP4A1 and CYP4A2, and the monoclonal specifically recognising CYP4A1 (Figure 3.3), CYP1A, both CYP1A1 and CYP1A2, CYP2B1 and CYP3A (Figure 3.4) subfamilies were seen to be elevated in both sexes in the diabetic state. In both sexes treatment of diabetic animals with insulin reversed these elevations; the CYP4A subfamily protein levels however were seen not to completely return to the levels seen prior to the onset of diabetes (Figure 3.3). These antibodies used in this study were raised against P-450 proteins purified from the rat and have been previously characterised and their specificity to the stated epitopes confirmed (CYP1A1, CYP1A2, CYP2B1 and CYP3A: Wolf & Oesch, 1983; Wolf *et al.*, 1984; CYP2E1: Hong *et al.*, 1989. CYP4A 1 and 2: Forrester *et al.*, 1990. CYP4A1 monoclonal: Savoy *et al.*, 1990).
The effect of the diabetic state on the mRNA levels of CYP2E1(Section 5.2) and CYP4A1 (Earnshaw *et al.*, 1988) subfamilies was investigated. Diabetes was seen to generate a marked increase in the levels of both the CYP2E1 and messages in both sexes in the liver. CYP2E1 mRNA levels are also elevated in the kidney in both sexes but elevation of CYP4A1 mRNA is only seen in the male kidney. The elevation of mRNA of both subfamilies was reversed by insulin treatment (Figure 3. 5, Figure 3.6).

Section 3. 5: The effect of acetone treatment on the protein and mRNA levels of the CYP2E1 and CYP4A1 enzymes

The use of acetone treated animals allowed the potential role of ketone bodies in the elevation in the levels of the CYP2E1 and CYP4A1 enzymes to be assessed. Acetone treatment produced a marked elevation in the level of the CYP2E subfamily protein in both the liver and the kidney in both male and female animals; this induction is reversed when the animals are allowed to recover from acetone treatment (Figure 3.7). These increases in CYP2E protein were not accompanied by increases in CYP2E mRNA levels in either the liver or the kidney (Figure 3.5). A decrease in the level of CYP2E1 mRNA was seen in the male kidney on treatment with acetone (Figure 3.5); several chemical inducers of the CYP2E subfamily have been reported to decrease the levels of CYP2E transcripts and a possible explanation for these observations is discussed in Section 3.7.5. By contrast the

protein and mRNA levels of CYP4A subfamily were unchanged by acetone treatment (Figure 3.6, Figure 3.8).

Section 3. 6: Control protein and mRNA markers for the diabetic and acetone treated animals, and the strain differences noted in the sexual dimorphism of several proteins between the BB and Wistar rats

Both the P-450 reductase and the microsomal glutathione S-transferase (μGST) were employed as microsomal protein markers and were seen to be unchanged by either the diabetic state or following exposure to acetone (Figure 3.9, Figure 3.10, Figure 3.11). The μGST mRNA was also seen to be unaffected by diabetes or acetone treatment (Figure 3.9, Figure 3.10). The μGST provides an invariant control protein and mRNA for studies of changes in xenobiotic metabolising P-450. Previous studies on the regulation of the μGST had shown that its levels were unchanged by compounds, such as 3-methylcholanthrene, which generate induction of several P-450 isoforms. Studies have suggested that factors regulating the level of μGST activity appear to do so via post-translational modification of pre-existing protein (Morgenstern & DePierre, 1988).

It is of note that a clear strain difference is present between the BB rats and the progenitor Wistar strain. A sexual dimorphism in the kidney of the μGST, present at both the mRNA and protein level in the Wistar animals with higher levels in the male than the female, is lost in the BB strain (Figure 3.9, Figure 3.10). Similarly a sexual dimorphism present in both the kidney and liver for the CYP4A subfamily, with again higher levels being seen in the male, is absent in the BB rats (Figure 3.3, Figure 3.6, Figure 3.7). A sexual dimorphism in the level of the CYP3A subfamily protein is however retained in the BB animals (Figure 3.4). A similar observation for strain-, and sex-related differences in xenobiotic metabolising enzymes has been noted previously. It was seen that the level of activity of *N*-acetylation was higher in the male BB/E rats than the female (Lindsay & Baird, 1990); in other strains the reversed relative levels of *N*-acetylase activity had previously been reported (Zidek & Janku, 1979). The basis for such differences in this instance is unclear but the observation that different rat strains, and other rodents, possess differences in P-450 and other xenobiotic metabolising enzymes is well established (Skett, 1987).

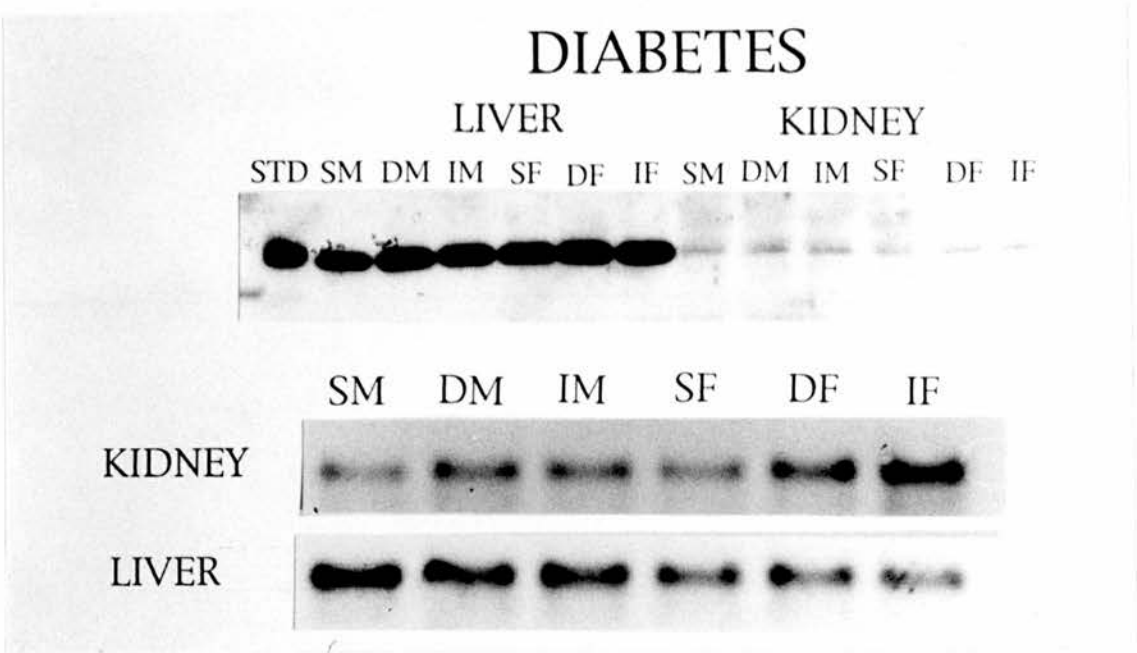


Figure 3.9: Western and Northern blot analysis of the effect of diabetes on the levels of the microsomal GST (μGST) protein and mRNA in the BB/E rat. Western blot (Upper): Microsomal protein (20μg of liver and 40μg of kidney) isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals were separated by SDS/PAGE on 12% gels. The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies raised to purified rat μGST protein. No bands other than those shown were detected and the μGST protein ran with a M_r of approximately 17,000. The μGST standard (**STD**) was prepared from the membranes of *E. coli* heterologously expressing the rat μGST protein. Northern blots (Lower 2): Total RNA was isolated from the tissues of animals treated as detailed above and 10μg of each RNA sample was subjected to horizontal gel-electrophoreses and transferred to a nylon filter by capillary action. The filters were hybridised with a radioactively labelled probe generated from a rat μGST cDNA; no bands other than those shown were detected and the μGST transcript ran with a size of approximately 950 bases.

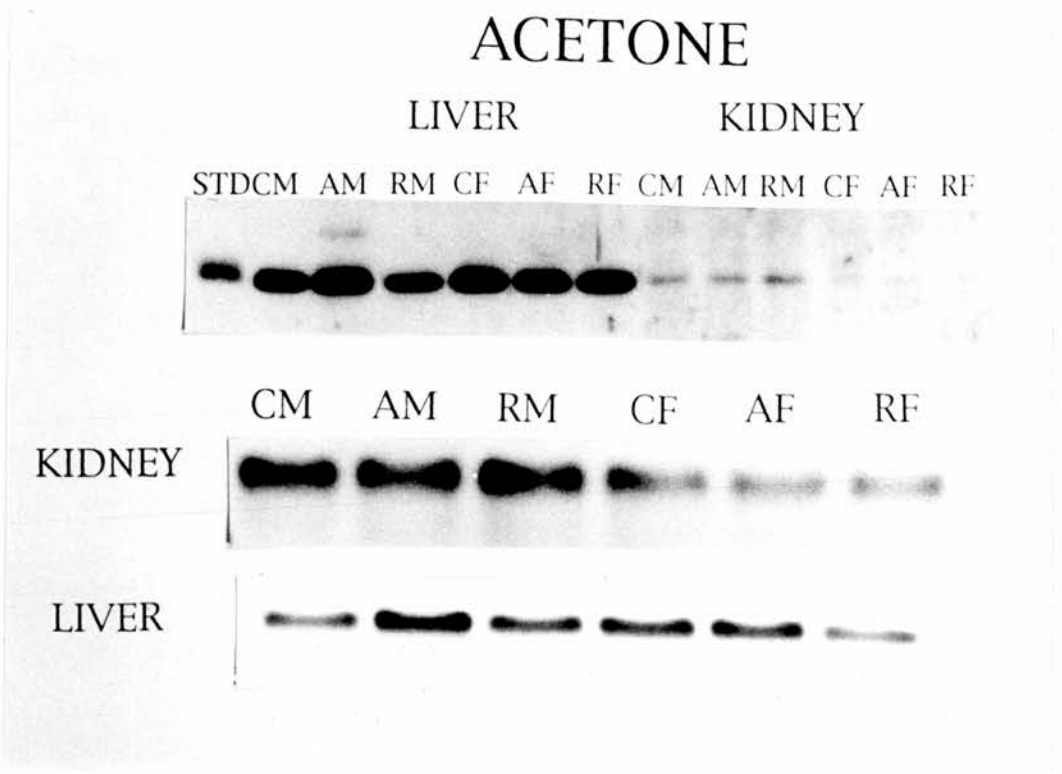


Figure 3.10: Western and Northern blot analysis of the effect of acetone treatment on the levels of the microsomal GST (μGST) protein and mRNA levels in the Wistar rat. Western blot (Upper): Microsomal protein (20μg of liver and 40μg of kidney) isolated from Wistar control male and female (**CM** and **CF**), male and female acetone treated by 1% acetone addition to drinking water (**AM** and **AF**) and animals allowed to recover from this treatment subsequently by one week with standard drinking water (**RM** and **RF**) were separated by SDS/PAGE in 12% gels.The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies raised to purified rat μGST protein. No bands other than those shown were detected.The μGST protein ran with a M_r of approximately 17,000. The μGST standard (**STD**) was prepared from the membranes of *E. coli* heterologously expressing the rat μGST protein. Northern blots (Lower 2): Total RNA was prepared from the tissues of animals treated as detailed above and 10μg of each RNA sample was subjected to horizontal gel-electrophoreses and transferred to nylon filters by capillary action. The filters were hybridised with a radioactively labelled probe generated from a rat μGST cDNA; no bands other than those shown were detected and the μGST transcript ran with a size of approximately 950 bases.

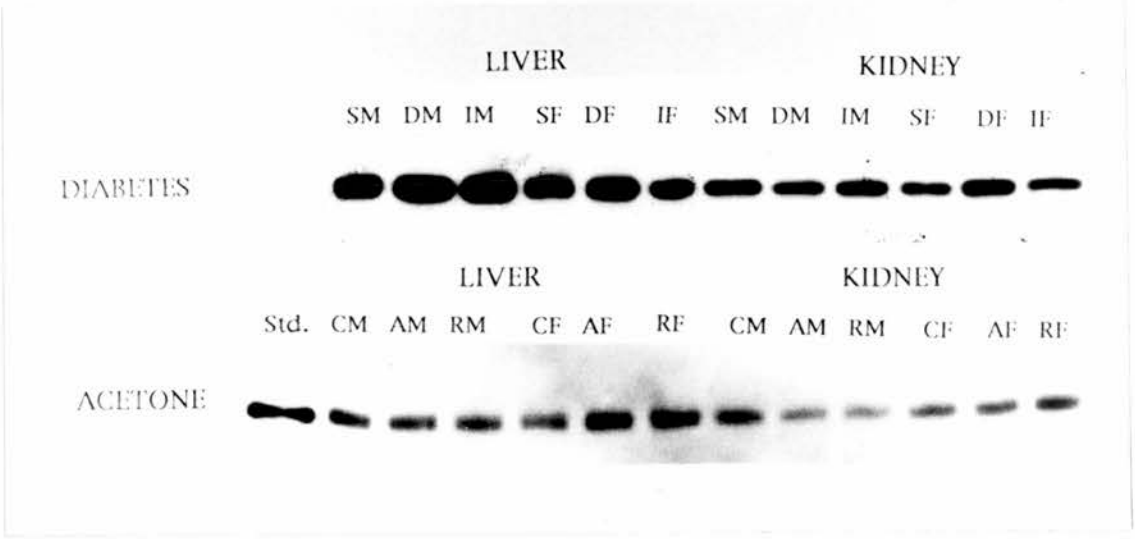


Figure 3.11: Western blot analysis of the effect of diabetes and acetone treatment on the protein levels of P-450 reductase in the BB/E rat and in the acetone-treated Wistar rat. Microsomal protein (10μg of liver and 20μg of kidney) isolated from subline male and female (**SM** and **SF** respectively), diabetes-detected, diabetes-prone male and female (**DM** and **DF** respectively), and insulin-treated, diabetes-detected, diabetes-prone male and female (**IM** and **IF** respectively) animals and the same quantity of microsomal protein isolated from Wistar control male and female (**CM** and **CF**), male and female acetone treated by 1% acetone addition to drinking water (**AM** and **AF**) and animals allowed to recover from this treatment by one week with standard drinking water (**RM** and **RF**), were separated by SDS/PAGE on 9% gels. The proteins were transferred to nitrocellulose filters and probed using polclyonal antibodies raised to purified rat P-450 reductase. The P-450 reductase protein has a M_r of approximately 100,000 and no bands other than those shown were detected. The P-450 reductase standard (**Std.**) was isolated from rat liver.

Section 3.7: What factors present in the diabetic state could account for the changes seen in P-450 enzyme levels

Although the levels of all the P-450 studied were seen to be modified by the diabetic state, the CYP2E and CYP4A subfamilies were more extensively studied and their potential endogenous inducers were analysed more fully. From this work, allied with previously noted changes in the diabetic state and the actions of chemical modulators of P-450 levels, the endogenous signals which may precipitate the changes seen in diabetes may be assessed.

Section 3.7.1: The diabetic modulation of the CYP2E subfamily mRNA and protein levels; a role for ketoacidosis

CYP2E has previously been shown to be induced by a variety of small solvent molecules; the endogenous signal for the induction in the diabetic state could therefore be the increased levels of ketones present in the blood as a result of increased fatty acid metabolism and decreased in glycolysis. Levels of β–hydroxy-butyrate were seen to rise approximately 22-fold and 15-fold in the blood of the diabetic female and male animals respectively (Table 3.1). The role of acetone in the induction of the CYP2E1 protein was demonstrated by the effects seen in the acetone-induced Wistar rats (Figure 3.7); this treatment however did not elevate the level of CYP2E mRNA (Figure 3.5) which was seen to be increased in the diabetic animals (Figure 3.5). Thus acetone may represent one of the endogenous signals modulating the levels of CYP2E in the diabetic state. Previous studies have indicated that acetone, and other small solvents metabolised by the CYP2E subfamily, have the capacity to induce the protein in this manner. This protein elevation has been suggested to be mediated by a process of substrate stabilisation of pre-existing CYP2E subfamily protein (Song *et al.*, 1989). The possibility also exists that acetone may accelerate the rate of CYP2E mRNA translation and degradation leading to lower CYP2E mRNA levels in chemically treated animals (Kim *et al.*, 1990; Kim & Novak, 1990). This possibility is discussed further in connection with the possible structural elements present within the CYP2E subfamily mRNA 5' untranslated regions (UTRs) (Section 3.7.5).

Section 3.7.2: The mechanism for the increase in CYP2E mRNA levels

The increase in the level of CYP2E mRNA could be the result of increased transcription, stabilisation of existing mRNA, or a combination of both these possibilities. Situations have been reported previously where both message stabilisation and increased transcription are seen. For example, in the control of phosphoenolpyruvate carboxykinase (PEPCK), a key enzyme in the control of gluconeogenesis, glucagon stimulated changes in intracellular cAMP levels and their associated phosphorylational changes, generate both an increase in the level of PEPCK transcription and an increase in message stability; these changes in the PEPCK transcript levels s are reversed by the action of insulin (Hod and Hanson, 1986; Petrsen *et al.*, 1989). In the only report attempting to address the molecular level at which the CYP2E transcript elevation occurs, nuclear run-on experiments were carried out from the CYP2E1 gene in hepatic tissue isolated from streptozotocin-treated rats. The data obtained from these experiments indicated that the CYP2E mRNA level increase seen was due to a stabilisation of pre-existing mRNA; no increase in CYP2E1 transcription was seen by comparison to the run-on results obtained from the β-actin gene (Song *et al.*, 1987). If this analysis is correct, then the mechanism for the elevation of the CYP2E subfamily transcript level in diabetes would generate a pleasing parallel to the proposed stabilisation of the protein also occurring in this state. However, results obtained in the present study of the control of the CYP2E subfamily in the diabetic state suggest that a transcript stabilisational does not occur, or, if it does it, may not account entirely for the increased levels of CYP2E mRNA seen.

Section 3.7.3: The use of β-actin as a probe for an invarient transcription rate in the diabetic state; the effect of the diabetic state on β-actin transcript levels in the BB/E rats

In suggesting that the CYP2E mRNA becomes stabilised in the diabetic state Song *et al.* (1987) based their findings on the assumption that the β-actin gene transcription levels would be unchanged either by the metabolic and hormonal changes experienced in the diabetic state, or resulting directly from the effects of streptozotocin treatment .
The effect of the diabetic state on β-actin transcript levels in twelve BB/E rat livers was studied; in this study the metabolic status of the animal was not revealed until after the analysis had been completed (i.e. a blind study). It is clear from this analysis that the level of β-actin is not unaffected but elevated by the diabetic state (Figure 3.12).

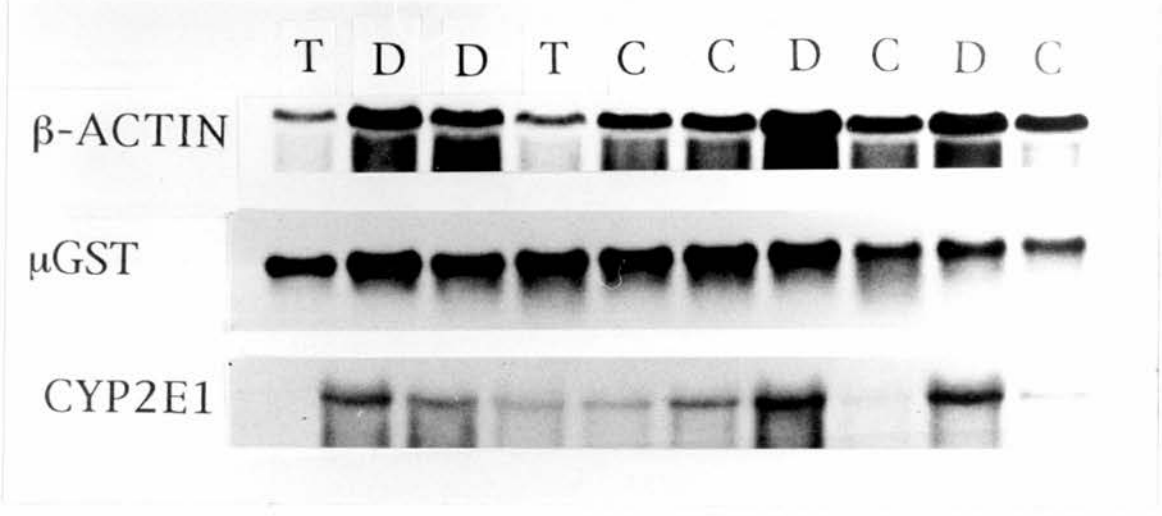


Figure 3.12: The effect of diabetes in the BB/E rat on the levels of β-actin transcripts compared to the effects observed in the microsomal GST (μGST) and CYP2E subfamily transcript levels. Total RNA was isolated from the livers of twelve BB/E animals, 10μg of total RNA was loaded per track, separated by horizontal gel electrophoresis and transferred to a nylon filter by capillary action. The filter was sequentially hybridised, and then stripped, with radioactively labelled probes generated from rat β-actin, rat μGST and mouse partial Cyp2e1 cDNAs. The nature of the source of the sample was then decoded to reveal the source of the tissue as indicated: **C:** Control subline. **D:** Diabetes-detected, diabetes-prone. **T:** Diabetes-detected, insulin-treated, diabetes-prone. The mRNA sizes ran with marker sizes of 1,000, 1,800 and 950 bases for β-actin, CYP2E and μGST respectively.

The nature of the rise in β-actin transcripts could be due either to transcriptional elevation or mRNA stabilisation and, if a similar change in the β-actin mRNA levels occurs in the streptozotocin treated animals, nuclear run-on experiments using β-actin as a presumed invariant control in isolation cannot distinguish between these two events. This observation means that the β-actin gene transcription rate cannot be used to gauge relative changes in the transcription levels of other messages in isolation.

Although β-actin is often regarded as a housekeeping gene has often been seen to be transcriptionally regulated. β-actin, and other cytoskeletal components such as α-actin and the tubulin gene family, have been shown to be transcriptionally regulated in a variety of situations including the actions of protein synthesis inhibitors (Greenberg *et al.*, 1986), serum stimulation (Ryseck *et al.*, 1989.), lectins (McCairns *et al.*, 1984), growth factors (Leof *et al.*, 1986) and insulin (Messina, 1992); transcriptional activation of β-actin is also seen to be correlated with cellular damage in certain cell types (Hoock *et al.*, 1991) where its trancriptional activity was decreased potentially by the actions of the nucleur phospho-protein p53.

Stabilisation of the CYP2E mRNA in the diabetic state cannot however be excluded as a result of this observation; analysis of features possessed by messages known to be regulated by stabilisation and the comparison of the nature of these features to those possessed by the CYP2 subfamily mRNA may reveal the possibility of mRNA stabilisation in the diabetic state.

Section 3.7.4: Regulating the stability of mRNA

If the CYP2E mRNA becomes stabilised in diabetes it is possible that structural comparisons can be drawn between it and other messages known to have regulated stability. mRNA is degraded from the 3' to the 5' end; the presence of a poly-(A) tail, and the subsequent protective action of the poly-(A) binding protein (PAbp), reduces the speed at which this occurs (Baer & Kornberg, 1983). Situations have been reported where the length of poly-(A) tails are modulated, for example growth hormone mRNA elongates in response to glucocorticoids (Paek & Axel,1987). This type of response has been seen to be mediated by the actions of secondary messenger systems, for example, factors which modulate protein-kinase C activity, leading to a change in the length of poly-(A) tail present on the corticotrophin releasing hormone mRNA (Adler *et al.*, 1992). Specific elements within the 3' untranslated (UTR) region of a message can also determine the rate of degradation, potentially by generating a competing binding site for pABP. Examples of messages with a stability of several days, for example haemoglobin, and several minutes,

for example c-*fos*, have been reported (Shaw & Kamen, 1986; Brawerman, 1989; Section 4.5.2).

These observations highlight the fact that a message is not a naked piece of RNA but a complex interaction between RNA and a variety of different proteins. An mRNA population is therefore divisible into two subpopulations; the first is being actively translated within ribosomes, the second is held within an inactive protein-bound messenger ribonuclear protein (mRNP) complex. The inactive mRNP component of a message population can account for up to 30% of the messages present within a muscle cell for example (Bag & Sarkar, 1985). The distribution between these two pools can be changed in response to a stimuli, for example, in the pancreatic islet cells at low glucose concentrations insulin messages are found mainly in the mRNP form however when stimulated by elevated glucose levels the mRNP becomes mobilised to the polysomes and translated (Welsh *et al.*, 1986). Most of the proteins present in these complexes, except PAbp, have not been well characterised and it appears that different proteins can either bind all mRNA molecules non-specifically or specifically bind only to a certain mRNA or group of mRNAs (Dreyfuss, 1986). The nature of the nucleases involved in the degradation of the messages is also not clear and whether a specific nuclease targets a certain mRNA or mRNA family, and is controllable in abstraction from the activity of other nucleases is uncertain. The existance of specific nucleases has been suggested, for example in the degradation of histone messages, although again this nuclease may just recognise messages lacking a poly-(A) tail (Ross *et al.*, 1987).

Many messages have been reported to become stabilised by the inhibition of protein synthesis; this observation suggests either that the nucleases responsible for degradation are rapidly turned over, or that to be degraded the message has to be translated and nucleases may be associated with the polysomes. It is clear therefore that there is an overlap between a messages stability and its translation rate; in many instances increased translation leads to accelerated degradation of a message and a block of translation leads to an increase in the stability of a message (Wisdom & Lee, 1991; Hua & Yaachov, 1992). The stability of a message is therefore the result of a complex series of interactions between structural features of the message, RNA binding and degrading proteins, and the translational machinery.

Section 3.7.5: Increased translation of the CYP2E subfamily mRNA after chemical induction

The translation of CYP2E mRNA has been reported to rise rapidly after treatment of animals with chemical inducers of the protein such as acetone (Kim & Novak, 1990, Kim *et al.*, 1990). Using sucrose density gradients it was shown that the CYP2E mRNA

population shifted to a higher density polysomal band after treatment with chemical inducers. This indicated that the number of ribosomes bound to each message increases on chemical treatment. In general it appears that translation may be limited by the initiation step, if the rate of initiation is increased, that is the translatability of the message increases, theoretically ribosomes can become stacked onto the message at a frequency of approximately one per thirty nucleotides. The stacking up of ribosomes on a message in this fashion leads to the shifting of the message to a higher density polysomal fraction (Wolin & Walter, 1988). In a separate study on the induction of CYP2E1 in the hamster it was suggested that ethanol treatment led to an increase in the level of CYP2E mRNA; the basis for this statement was the observation that ethanol treatment led to an increase in the ammount of CYP2E1 protein produced by a specific quantity of total RNA used to programme a reticulocyte-lysate translation system (Kubota *et al.*, 1988). It is however also possible to interpret these observations by suggesting that the level of translatability of the CYP2E1 messages present, as opposed the total amount, has become elevated by the chemical treatment as no direct quantification of the CYP2E1 mRNA was carried out.
The level of translatability of a message is dependent on a complex interaction of structural features of the message itself and other protein components. Factors affecting the level of translation of a message, or its "specific activity" or "strength", include the type and context of the initiation codon as well as the effect of proteins binding recognition sites, possibly contained within RNA folding structures, both within and outside the coding region of the message (Galili *et al.*, 1988).

Section 3.7.6: An example of regulatable mRNA stability and translatability: ferritin and transferrin receptor mRNA in iron homeostasis

The potentially damaging effect of free radicals generated by iron stored within cells mean that a precise control of cellular iron levels must be maintained. Iron is stored in association with the protein ferritin and is taken up into cells, in association with its transport protein transferrin, by the transferrin receptor. In situations of high iron concentration levels of ferritin synthesis rise to increase iron storage via a ten to forty fold increase in the translatability of pre-existing ferritin mRNA, actually the product of two highly related genes. Conversely the level of iron uptake falls due to a twenty fold decrease in the stability of the transferrin-receptor mRNA stability. The reverse situation occurs in the presence of low iron or following addition of iron chelators such as desferrioxamine (Rao *et al.* , 1986, Bridges & Cudkowski, 1989).
Deletion analysis of the transferrin-receptor mRNA revealed that the portion of the message responsible for the regulatable stability was present within the 3' untranslated region (UTR) and consisted of sixty nucleotides which generated, when sub-optimally folded in a

computer analysis, 5 firm stem-loop structures. The transferrin-receptor mRNA is stabilised by the regulatable binding of a protein which recognises sequence motifs within structural features at the 3' end of the message (Owen & Kuhn, 1987). Deletion analysis of the ferritin messages revealed a role for sequences within the 5' UTR necessary for the generation of regulatable translatability of the messages. The 28 nucleotide region of the 5' UTR which varied the message translatability was capable of forming a similar predicted stem-loop secondary structure to those seen at the 3' UTR in the transferrin-receptor mRNA. This feature of the 5' UTR was seen to be conserved in the ferritin messages of all the species studied (Hentz *et al.*, 1987; Roualt *et al.*, 1989). Translational initiation of the ferritin message is blocked in the presence of iron by the binding of the regulatory protein to the 5' UTR motif; in the absence of iron the 5' UTR/protein interaction is lost and the mRNA shifts from mRNP to the polysomes where it is translated (Brown *et al.*, 1989; Haile *et al.*, 1989).

Thus, in a reciprocal manner, a regulatable RNA-binding protein can bind structural features contained within the untranslated regions of a message and modify either the rate of translation or degradation operating on the message. In this instance the presence of the protein binding 3' UTR of the transferrin-receptor mRNA generates increased stability of a message, or, as is seen in the ferritin messages, binding at the 3' UTR blocks the translation of the message. In a similar manner computer prdicted stem-loop structures have been defined within the 3' UTR of histone messages, again widely conserved throughout evolution, which lead to the generation of regulatable stability of these messages in S-phase (Levin *et al.*, 1987). A similar observation has been made in connection with the regulated stability of the phosphoenolpyruvate carboxykinase mRNA where it was seen that motifs within the 3' UTR conferred increased stability to the message in the presence of glucocorticoids (Petersen, *et al.*, 1989).

It is not always the case however that features controlling message stability or translatability are contained within the UTR regions. Tubulin mRNA stability is regulatedby a feedback mechanism dependent on the levels of ambient tubulin heterodimers within a cell. These heterodimers interact with the first four amino-acids at the N-terminal of the protein as it becomes translated and, potentially either by causing the translation process to slow and so giving more opportunity for degradation, or by activation of a nuclease associated with the ribosome, increase the degradation rate of the tubulin message. In this instance however, where the controlling features for a message's stability are contained within the coding region, the fact that the proteins involved naturally oligermerise may indicate that such control mechanisms are unique to proteins capable of self-recognition via oligermerisation (Patcher *et al.*, 1988; Yen *et al.*, 1988). More generally portions of a message contributing to regulation are contained within the UTRs and, being free from amino-acid coding

restraints, can be used to generate specific secondary structures and contain recognition motifs to allow binding of regulatory proteins.

Section 3.7.7: Does the CYP2E subfamily mRNA possess features which may allow the modulation of degradation or translation

Molecular evidence suggesting the possibility of regulatable translation or degradation of the CYP2E subfamily mRNA

One factor governing the rate of translational initiation of a message is determined by the nucleotide context of its first AUG codon. The presence, or absence, of certain nucleotides in proximity to the initiation codon plays an important role in determining the level of translational initiation from a message. The presence features within the 5' UTR, with the possibility of formation of structures and content of sequence motifs interacting with regulatable protein components, as seen with ferritin mRNA, also may allow the control of initiation.
The initiation AUG codons of the CYP2E subfamily messages are all in near perfect consensus contexts (Kozak, 1987) suggesting that the CYP2E messages are potentially readily translated (Table 3.2).

Table 3.2: The context of the initiation methionine codons of the CYP2E subfamily messages known to date compared with the derived mammalian consensus (Con.) AUG context.

Rabbit	A	A	G	C	A	C	C	**A**	**U**	**G**	G	C
Human	C	G	G	C	A	C	C	**A**	**U**	**G**	U	C
Rat	U	G	G	C	A	C	C	**A**	**U**	**G**	G	C
Mouse	C	G	G	C	A	C	C	**A**	**U**	**G**	G	C
Con.	C	G	G	C	A	C	C	**A**	**U**	**G**	G	C

The 5' UTR of the human (Umeno *et al.*, 1988a), rat (Umeno *et al.*, 1988b) and rabbit (Khani *et al.*, 1988) CYP2E messages were determined by S1 mapping and primer extension assays showing the messages to have a leader UTR of around fifty nucleotides. Alignment of the 5' UTR, and by homology the mouse Cyp2e1 UTR, and computer generated folding of these sequences to their lowest energy state form allowed the determination of folding structures which may be adopted by the messages (Section 5.4.4, Figure 5.7a). The generation of these folding patterns indicated that a highly conserved secondary structure could be generated by the 5' UTR of the CYP2E subfamily messages;

sequence analysis of this portion of the CYP2E subfamily message suggested the presence of a highly conserved NFκβ recognition site which could potentially recognised by the series of DNA binding proteins (Section 4.13; Section 4.13.1; Figure 5.7b). Some of the proteins which have been shown to bind NFκβ recognition sites contain zinc-finger motifs potentially capable of binding both RNA and DNA (Baldwin *et al.*, 1990; Dressler & Gruss, 1988). The presence within the 5' UTR of the CYP2E subfamily messages of these highly conserved structural elements, and possible protein recognition motifs, suggests that a mechanism for the regulation of translation could be mediated by factors binding to this portion of the message in a similar manner as discussed in connection with ferritin mRNA translation control (Section 3.7.6).

The level of conservation in the 3' UTR of the CYP2E subfamily messages is low (Section 5.4). This observation suggests that the 3' UTR of the CYP2E subfamily messages may not contain ragulatable degradation conferring motifs as seen in the transferrin receptor mRNA 3' UTR (Section 3.7.6). This suggestion is stressed by the observation that the mouse Cyp2e message was seen to have lost its original 3' UTR sequence following the insertion of a β_2 repeat element. The removal of the original Cyp2e mRNA 3' UTR by the β_2 repeat element does not however block the induction of the message by starvation in the mouse (Section 4.5). That Cyp2e1 mRNA induction is still seen in the starved mouse suggests that the 3' UTR plays no function in regulation of the Cyp2e mRNA and so potentially the other CYP2E messages. If stabilisation of the CYP2E subfamily messages were to occur in diabetes then these observations collectively suggest that it would have to involve regions outside the 3' UTR; with the exception of the autoregulation of oligermeric self-recognising proteins (Yen *et al.*, 1988) no evidence for stabilisation of a message by a region other than the 3' UTR has been reported.

The combined observations that β-actin transcript levels are elevated in the diabetic state and so cannot form a reliable invariant control transcript and that there is apparently no role for a conventional mechanism involving the 3' UTR in the starved mouse, suggests that a role for stabilisation of the CYP2E subfamily in the diabetic state is questionable. Such a control mechanism and may not account for the changes in the levels of CYP2E transcripts seen. It is possible however that there may be a role for a increase in translatabilty of the CYP2E subfamily message in the diabetic state potentially mediated by features within the conserved 5' UTR.

Section 3.7.8: Factors previously seen to modify CYP2E mRNA levels in the rat and their potential role in the diabetic induction of this subfamily; The role of growth hormone in the regulation of the CYP2E sufamily in the rat

Growth hormone has previously been shown to modulate the levels of rat CYP2E1 mRNA; it is perhaps important to note therefore that growth hormone levels fall in the diabetic rat (Tannenbaum *et al.*, 1981. Tannenbaum, 1981). Hypophysectomy, leading to a loss of growth hormone, has been demonstrated to affect the levels of several P-450s in the rat. The levels of CYP1A, the CYP2B subfamily and CYP2E1 were seen to be elevated in the hypophysectomised rat; CYP2E levels were seen to be elevated by both biochemical and immunological assay following hypophysectomy and the removal of growth hormone (Williams & Simonet, 1988; Yamazoe *et al.*, 1989a). The hepatic content of CYP2E1 mRNA was seen to increase between two and five-fold and the levels were returned to normal after administration of growth hormone to the hypophysectomised rat (Yamazoe *et al.*, 1989a). As previously noted in earlier biochemical studies of P-450 changes in diabetic rats the levels of certain sex dependent P-450 change (Warren *et al.*, 1983), for example the level of CYP2C11 was seen to fall with the onset of diabetes in the male rat (Favreau *et al.*, 1987a).

The effects of growth hormone and its mechanism of action on the P-450 genes which it regulates have been extensively studied in the rat CYP2C gene family. Some members of the rat CYP2C subfamily are sexually dimorphic and some are sex-dependent. CYP2C11 (associated with testosterone 16 α-hydroxylase activity) and CYP2C13 (associated with testosterone 6 β-hydroxylase activity) are male specific, whereas CYP2C12 (steroid sulphate 15 β-hydroxylating) is female specific. CYP2C7 (retinoic acid and vitamin A metabolising) is a constitutive but sexually dimorphic enzyme with around a three-fold higher activity in the female. Hypophysectomy suppresses the expression of CYP2C11 in males, CYP2C12 in females and CYP2C7 in both sexes. The level of expression of CYP2C11 and CYP2C13 however become elevated in the female to ten and fifty percent of those present in the male (Mode *et al.*, 1988; McClellan *et al.*, 1989; Westin *et al.*, 1990;Zaphiropoulos *et al.*, 1990). The nature of CYP2C gene elevations were studied by nuclear run-on assays using both β-actin and tyrosine amino-transferase gene transcription as controls, and were shown to be mediated transcriptionally (Legraverend *et al.*, 1992). A fall in the level of CYP2C11 occurred in both hypophysectomised and diabetic male rats; it is possible that in both these situations the signalling pathways modulated to produce the changes in transcript level are the same and therefore in both cases the changes may be mediated transcriptionally. Growth hormone treatment of the diabetic male animal however did not reverse the decrease in CYP2C11 mRNA whereas insulin treatment did (Yamazoe *et al.*, 1989b). In a separate study it was reported that diabetes leads to resistance to the

actions of growth hormone and the levels of the growth hormone receptor are reduced in the diabetic state in rats suggesting that insulin allows a growth hormone signalling pathway to operate (Baxter *et al.*, 1980).

A synergistic action between insulin and growth hormone may therefore produce transcriptional changes in the level of certain P-450 in the rat in diabetes. Problems with this suggestion however arise when the both the effect of diabetes on the level of growth hormone, and the effect of growth hormone on P-450s, in other species are examined. In the human it was seen that the levels of growth hormone were elevated in starvation and the diabetic state (Ho *et al.*, 1988; Asplin *et al.*, 1989) yet the level of CYP2E1 protein was observed to be elevated in the lymphocytes of diabetic patients (Song *et al.*, 1990). Studies in the mouse, using both hypophysectomised and genetic models to remove circulating growth hormone, have shown that the lack of growth hormone does not produce any change in the levels of a variety of P-450s in this species (Henderson *et al.*, 1990; Hong *et al.*, 1990. Section 4.6) yet starvation was seen to elevate the Cyp2e subfamily protein (Section 4.5.2). These observations suggest either that in diabetes rat P-450 genes are controlled by changes in growth hormone levels and that elevations seen in the human CYP2E subfamily in diabetes, and the mouse in starvation, are coincidental and arrived at by another mechanism, or that common controls, not involving growth hormone mediate the P-450 elevations seen in diabetes. Differences in the potential transcriptional control elements inthe rat and CYP2E subfamily gene and the mouse have been identified which may allow regulation by growth hormone to occur in one species alone (Section 4.12). However, n the light of the observed cross species conservation in the induction of the CYP2E subfamily in diabetes and starvation, it would seem logical to suggest that changes in growth hormone levels, which are not conserved between species in these states, may not be the sole effectors of the changes in CYP2E subfamily transcript levels in these states.

Section 3.8: The factors contributing to the modulation of the CYP4A subfamily in diabetes

The CYP4A subfamily was seen to be elevated in the diabetic state at both the mRNA and protein level (Figutre 3.3, Figure 3.6). Changes in the diabetic state, either hormonal or metabolic, must therefore be responsible for these changes and produce them as a result of the actions of endogenous signalling pathways.

Section 3.8.1: The role for diabetic ketosis in the induction of CYP4A

Treatment of Wistar rats with 1% acetone in their drinking water had no effect on the level of CYP4A protein or mRNA (Figure 3.8, Figure 3.6). Thus the CYP2E protein induction seen on acetone treatment appears likely to operate via a substrate linked stabilisation mechanism rather than a process which, via an acetone associated or activated pathway, P-450s in general become stabilised. This suggests that other components of the metabolic and hormonal changes occuring in diabetes may be responsible for the endogenous signals leading to CYP4A subfamily induction.

Section 3.8.2: Factors previously seen to induce the CYP4A subfamily: peroxisome proliferators

Xenobiotics capable of inducing members of the CYP4A subfamily have been seen to include a group of hypolipidaemic drugs such as clofibric acid and its structural analogues nafenopin, methlyclofenapate and WY-14,643, as well as industrial plasticizers such as phthalate esters, for example di-(2-ethyhexyl)-phthalate, and certain herbicides, for example 2,4,5 trichloropheno-oxyacetic acid. Collectively all these chemicals have the capacity to generate peroxisomal proliferation and are therefore termed "peroxisome proliferators". Treatment with clofibrate, and other peroxisomal proliferators, induces the diagnostic activities associated with a series of enzymes responsible for the cyanide-insensitive β-oxidation pathway within the peroxisomes (Reddy *et al.*, 1986). The hypolipidaemic drugs mediate their action by generating a proliferation of peroxisomes and so accelerating fatty acid metabolism leading to hypolipidaemia. Broadly therefore, in physiological terms, the actions of peroxisome proliferators could be likened to the effects of a fall in the insulin:glucagon levels leading to an acceleration of the use of free-fatty acid as a fuel (Section 3.3). These compounds have become paradigms for non-genotoxic xenobiotics, i.e. they generate cellular damage indirectly potentially through the elevation of oxidative stresses within the cell as the level of peroxisomal activity becomes induced (Reddy *et al.*, 1992).

Increased levels of both CYP4A protein, ω and ω-1 hydroxylation of lauric acid (the diagnostic activies of the CYP4A subfamily), and mRNA were observed in the livers of clofibrate-treated rats (Hardwick *et al.*, 1987). Nuclear run-on assays, using CYP3A and CYP2B subfamily members previously shown to be unaffected by clofibrate treatment as invariant controls, showed that the induction was mediated transcriptionally (Hardwick *et al.*, 1987). The actions of hypolipidaemic drugs appear to be localised predominantly to the liver. Studies on the level of renal induction of the CYP4A subfamily revealed that, although CYP4A is present as a high percentage of the total P-450 content in the kidney

(around 30% in the kidney, but only around 5% in the liver), the kidney is less responsive to the inducing effects of peroxisomal proliferators (Sharma *et al.*, 1989).

Section 3.8.3: The mechanism of action of peroxisome proliferating xenobiotics

The broad effect of peroxisomal proliferators could be likened to the induction of the use of free-fatty acids as a fuel supply in the fasted, starved or diabetic states as the insulin: glucagon ratio falls. It is therefore possible that peroxisomal proliferators are utilising an inductive signalling pathway normally stimulated by physiological agents. The processes by which the peroxisome proliferators operate may therefore, in potentially mimicking the actions of endogenous stimulators, give an insight into the processes that lead to CYP4A induction in the diabetic state.

DNA binding proteins capable of acting as effectors for the actions of peroxisomal proliferators have been isolated. The first was found through the probing of a mouse cDNA library with oligonucleotides generated to the conserved binding regions of the steroid hormone superfamily of receptors. The compounds capable of activating the steroid hormone receptor proteins encoded by the clones obtained were analysed through the fusion of the ligand binding domain of the novel receptors to the DNA binding domain of receptors, glucocorticoid and oestrogen, with known DNA target sequences. Using this assay, in conjunction with the target DNA sequences upstream of a chloramphenicol acetyl-transferase (CAT) transcript in mammalian tissue culture, the chemicals capable of activating the chimeric receptors were analysed. One such receptor, with a M_r of around 52,400, was activated by peroxisomal proliferators and was termed the mouse peroxisomal proliferator activated receptor (mPPAR). A good correlation between the ability of a compound to generate peroxisome proliferation *in vivo* and the strength of the CAT response was noted. The peroxisome proliferators did not however bind the receptor itself directly strongly suggesting that they mediated their actions indirectly via another signalling pathway (Isseman & Green, 1990).

A similar study leading to the cloning of a rat analogue, the rPPAR which has 97% similarity to the mPPAR, studied more inducing chemicals in an attempt to isolate the physiologically relevant activator. These studies concentrated on free-fatty acids such as arachidonate, laurate, and linolate and their metabolic intermediates as potential endogenous ligands for the PPAR. These potential ligands again, as seen for the peroxisome proliferators, did not bind the PPAR with the degree of specificity that might be expected from analysis of other members of the steroid hormone receptor superfamily. The presence of an easily generated, but poorly metabolised, acidic group in the compounds was the only common structural feature identified in the potential ligands (Gottlicher *et al.*, 1992). What seems to be a likely candidate for the position of the PPAR ligand is a compound generated

in response to common intermediates in the metabolism of fatty acids. The ligand may be generated by lipid associated signalling pathways, such as those generated by arachidonic acid, and the biologically active intermediate may act either as the PPAR ligand itself or lead to the generation of post-translational modification of the PPAR allowing it to bind the true ligand (Gottlicher *et al.*, 1992). The direct action of free-fatty acids as low avidity ligands however cannot be ruled out totally and it has been observed that poorly metabolised free fatty acids as well as increased levels of free-fatty acids *per se* act as peroxisome proliferators *in vivo* (Berge *et al.*, 1989; Aarsland *et al.*, 1989).

More members of the PPAR subfamily have been identified. A PPAR cDNA has been cloned from human (Schmidt *et al.*, 1992) and three PPAR cDNAs cloned from *Xenopus* (Dreyer *et al.*, 1992). The three receptors cloned from *Xenopus* are developmentally regulated and are capable of inducing CAT activity in a non-chimeric form from the 5' flanking region of acyl-CoA oxidase gene, a component of the peroxisomal β-oxidation pathway seen to become transcriptionally activated*in vivo* by peroxisomal proliferators (Dreyer *et al.*, 1992). In a similar manner regions of the 5' of the rat peroxisomal enoyl-CoA hydratase/3-hydroxy acyl CoA dehydrogenase gene involved in the response to peroxisome proliferators has been identified (Zang *et al.*, 1992).

Many other members of the steroid-hormone receptor superfamily have been isolated in a manner similar to that for the PPAR but very few ligands have been identified suggesting perhaps that these "orphan" receptors may participate in intracellular signal responses and that their ligands are generated intracellularly as a result (O'Malley, 1990).

A peroxisomal proliferator binding protein (PPbp) was purified from the cytoplasm of rat livers using affinity columns containing immobilised peroxisomal proliferators. The PPbp had a M_r of around 70,000, far greater than that of the PPAR and is thought to exist as a dimer in the cytoplasm (Lalwani *et al.*, 1987). The relationship between the PPAR and the PPbp is not clear, the PPAR was activated by the hypolipidaemic WY 14,643 whereas the PPbp did not bind this compound for example; these results suggest that potentially more than one signalling pathway may be employed by the peroxisomal proliferators or the PPbp may be the binding protein *per se* for the xenobiotics and endogenous signals are then generated which go on to generate the PPAR ligand.

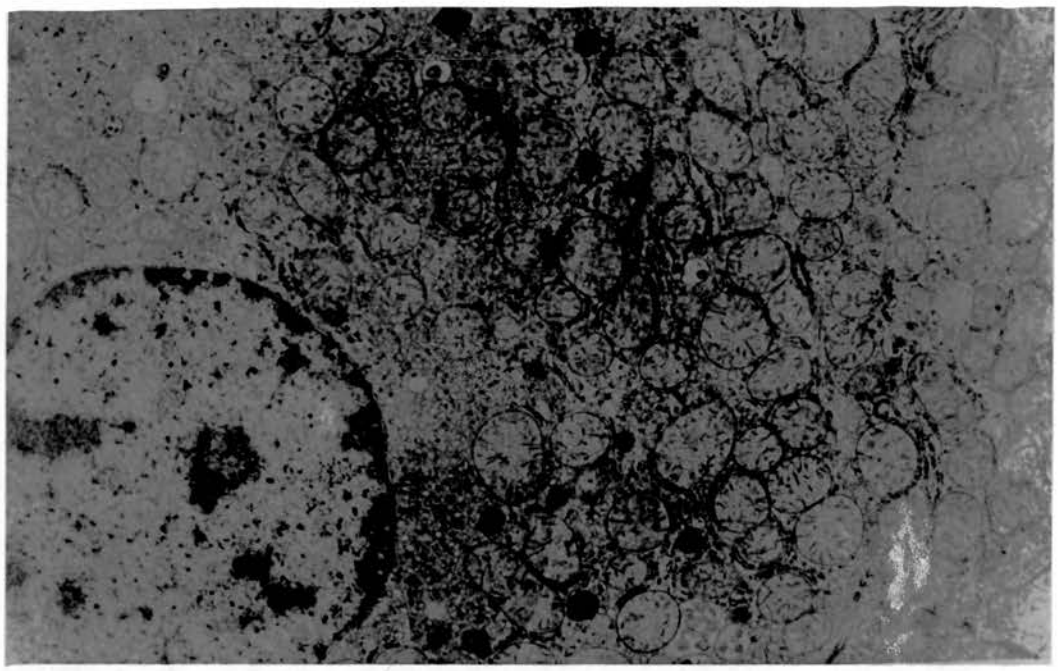
It is also not clear if the peroxisome proliferators generate the actual peroxisome proliferation seen. The transcriptional induction of components of the cyanide-insensitive peroxisomal β-oxidation pathway and the induction of actual peroxisomes has been seen to be temporally separated. Benzafibrate induces the level of β-oxidation enzymes but not the actual quantity of peroxisomes in the rat (Lazarow *et al.*, 1982) and similarly, although inducing the β-oxidation components, clofibrate did not induce peroxisomes in *Xenopus* (Ciolek & Dauca, 1991). Peroxisome proliferators have now been shown to transcriptionally activate the 5' regions of the CYP4A sufamily in association with the

PPAR. In luciferase reporter experiments in mammalian tissue culture the rabbit CYP4A6 gene 5' region was activated by clofibrate in the presence of a co-transfected PPAR; the DNA elements found to be necessary for this induction were found to be highly similar to those of the previously characterised fatty-acyl CoA oxidase gene (Muerkoff *et al.*, 1992). This suggests that the processes involved in the action of peroxisome proliferators, and so potentially the endogenous activators, in the induction of the CYP4A subfamily operate. One striking feature of their action is the co-ordinate induction of a series of genes which contribute to the acceleration of peroxisomal β-oxidation.

Section 3.8.4: Changes in peroxisomal activity in the diabetic BB/E rat

If similar pathways for induction of the CYP4A subfamily are used by both the peroxisome proliferators and the diabetic state then potentially the co-ordinate activation of cyanide-insensitive β-oxidation and peroxisomal proliferation may also occur. Both the levels of cyanide-insensitive β-oxidation and the ultra-structure of the BB/E diabetic rat livers were investigated to see if such a co-ordinate fatty acid metabolism response is associated with the CYP4A induction seen in diabetes. An increase in the level of cyanide-insensitive β-oxidation was seen in the diabetic animals suggesting that the components of the peroxisomal β-oxidation pathway, seen to be activated in a co-ordinate manner by peroxisomal proliferators, also become activated in diabetes (Table 3.3). An increase in the percentage of the cytoplasm occupied by peroxisomes in the liver cells, as an indication of peroxisomal proliferation (Table 3.3, Figure 3.13), was however only markedly seen in one diabetic animal suggesting that a separation in the activation of β-oxidation and peroxisomal proliferation may occur in diabetes. A similar observation had been made in chemically treated rats where, although an increase in peroxisomal activity was seen, no actual peroxisomal proliferation occurred (Lazaro *et al.*, 1981; Ciolek & Dauca, 1991). Potentially more marked peroxisomal proliferation may be produced if it were possible to extend the period which the animal remains diabetic; this however is not possible as, without insulin administration, the animals die soon after the sampling times of the animals used in this study (Figure 3.13).

A.



B.

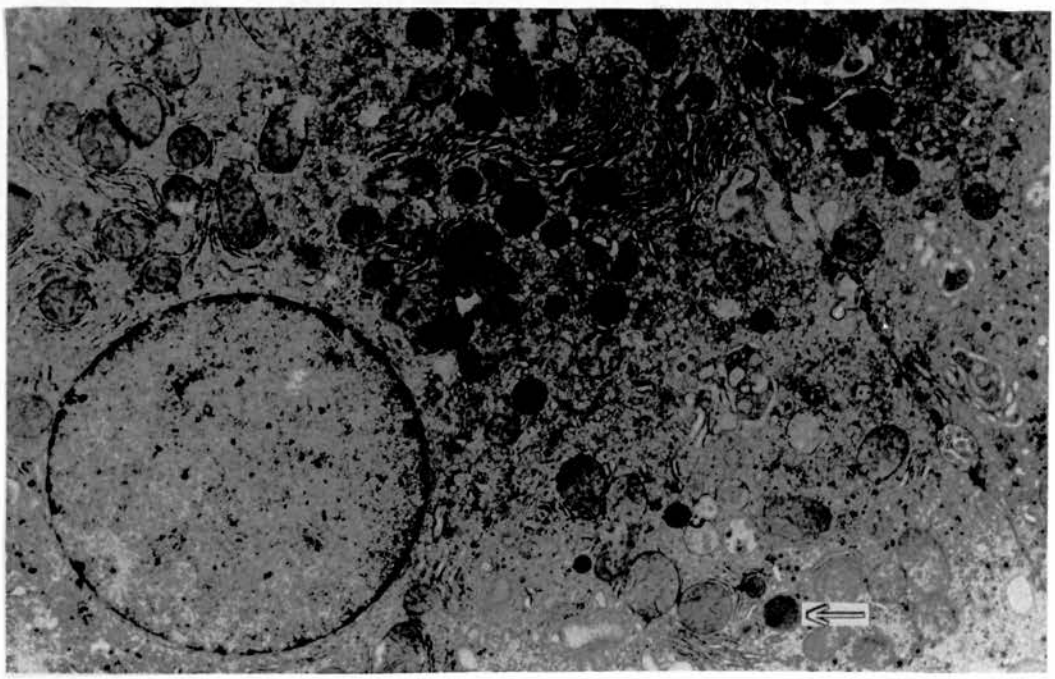


Figure 3.13: Transmission electron microscopic (TEM) analysis of the level of peroxisomes present in the hepatocytes of the BB/E rats. Liver samples taken from the BB/E animals were fixed and analysed by TEM to a magnification of X 8,300. **A:** Analysis of the level of peroxisomes present in the hepatocyte cytoplasm of a diabetes-resistant, subline, BB/E male rat. **B:** Analysis of the hepatocyte cytoplasm of the one male BB/E diabetes-detected, diabetes-prone male rat in which an increase in peroxisomal volume in the cytoplasm was noted. Peroxisomes are seen as dark staining microbodies as indicated by the arrow in B. This analysis was carried out in collaboration with Dr J. Foster and Dr C. Bowling, Zeneca Central Toxicology Laboratories, Cheshire.

Table 3.3: The changes in the rates of cyanide insensive β-oxidation and cytosolic peroxisomal content in the BB/E rat study.The rate of NAD reduced (nmoles/min/mg of liver sample) in the presence of cyanide is an assay for the activity of palmitoyl CoA dependent reduction of NAD^+ and so reflects the level of peroxisomal β-oxidation (Bronfman *et al.*, 1979); assays were performed in collaboration with Dr C. Elcombe and Dr S. Tittensor, Zeneca Central Toxicology, Cheshire. The percentage peroxisomal content in the centrilobular hepatocytes was estimated on X 8,300 transmission electron micrographs using a 345 intercept square lattice (Moody & Reddy, 1976); analysis was performed in coaboration with Dr J. Foster and Dr C. Bowling, Zeneca Central Toxicology, Cheshire.

	nmol reduced NAD per min, per mg protein.	peroxisome % in the cytoplasm
SUBLINE MALE	2.33	1.55
	2.77	2.13
	1.85	
DIABETIC MALE	3.61	2.50
	4.25	6.66
INSULIN TREATED MALE	1.84	3.07

Section 3.8.5: The endogenous signals potentially inducing the CYP4A subfamily in diabetes

In the diabetic state the activation of lipolysis and the switch to the use of free-fatty acids as the insulin:glucagon levels fall leads to the elevation of free-fatty acid levels in the blood. From the studies into the potential effectors of peroxisomal proliferator action it is possible that an increase in free-fatty acid, or triglyceride levels, contributes either directly or indirectly to the activation of the PPAR and PPbp. As a result of these stimuli the activated PPAR may transcriptionally activate the CYP4A genes (Muerhoff *et al.*, 1992) and it has been shown to transcriptionally activate the acetyl-CoA oxidase (Dreyer *et al.*, 1992) and enoyl-CoA hydratase/3-hydroxy acyl-CoA dehydrogenase (Zang *et al.*, 1992) components of the peroxisomal β-oxidation pathway. The putative endogenous signal for the elevation of CYP4A may therefore be either the elevated levels of triglycerides, seen to be increased

approximately 3-fold and two-fold in the male and female animal respectively, or free-fatty acids, seen to be elevated approximately 3-fold or 2-fold in the male and female animal respectively (Table 3.1), or metabolites with biological activity arising from these elevations.

The fact that little change was seen in the levels of CYP4A in the diabetic kidney suggests that the same pathway leading to the induction of the CYP4A subfamily by the the peroxisome proliferators, which produce little CYP4A response in the kidney, (Sharma *et al.*, 1989) may be operating in diabetes. The seeming refractory nature of extra-hepatic tissues to the effects of peroxisomal proliferators appears as a common feature shared by other peroxisomal-associated genes activated by their action in the liver. It was seen that components of the peroxisomal β-oxidation pathway were unaffected by clofibrate treatment in the rat kidney despite being highly induced in the liver (Nemali,*et al.*, 1988). This may reflect potentially a lower concentration of signalling components of the activating pathway machinery within the kidney and this observation is in marked contrast to the increased levels of CYP2E mRNA and protein in the diabetic kidney. This observation suggests that potentially two distinct endogenous mechanisms are responsible for the elevation of the CYP2E and CYP4A subfamilies.

Section 3.9: The endogenous signals responsible for the changes in the levels of the other P-450 seen to be modulated in the BB/E rat

Protein level increases were seen to occur in the diabetic livers of the CYP1A, CYP2B and CYP3A subfamilies. As has been suggested for the CYP2E and CYP4A subfamilies a combination of metabolic and hormonal changes may be responsible for the generation of the changes seen suggesting the presence of endogenous control mechanisms for these components of the drug metabolising system. Proteins in the CYP1A subfamily have previously been noted to become elevated in rats by treatment with high levels of tri-acyl glycerol, leading to elevated ketone body levels (Barnet *et al.*, 1990); this suggests a role for acetone in the CYP1A protein induction seen in diabetes. Similarly increased levels of acetone have been shown previously to generate increases in the levels of members of the CYP2B subfamily (Johansson *et al.*, 1988). A comparison of the regulation of the CYP2E, CYP2B and CYP1A subfamilies in the diabetic state, in terms of the potential role of acetone as an endogenous controller, can be drawn. Hypophysectomy generated an elevation in the level of the CYP2B subfamily suggesting that growth hormone has a negative effect on its level of expression in the rat (Yamazoe *et al.*, 1987). Hypophysectomy however produced no changes in the levels of the CYP1A subfamily suggesting therefore that a growth hormone level decrease in the diabetic state would leave the CYP1A unaffected (Yamazoe,*et al.*, 1987). Hypophysectomy has also been shown to

increase the level of the CYP3A subfamily in both rats and mice (Meehan *et al.*, 1988; Lemoine *et al.*, 1988). The possible species differences associated with these responses has been discussed in connection with the CYP2E subfamily (Section 3.7.8).

Section 3.10: Potential roles for the P-450 levels modulated by endogenous signals in the diabetic state

If the changes seen in the P-450 profiles are induced through the actions of endogenous signalling pathways then such changes may be generated for a reason and may not just be the inadvertent results of metabolic and hormonal perturbations in the diabetic state. Potential roles, unrelated to the metabolism of exogenous xenobiotics, for some of the xenobiotic metabolising P-450 enzymes may therefore be present and these may provide a rationale behind the induction of these enzymes in diabetes and so potentially fasting and starvation.

Section 3.10.1: A role for the CYP2E subfamily in acetone metabolism in diabetes and starvation

Acetone, produced as a result of incomplete use of the products of fatty acid oxidation, is now thought to be further metabolised and not, as previously believed, just accumulate prior to passive excretion in the breath, urine and sweat. Although some tissues, such as the brain, can adapt to use ketone bodies as an energy source, the potential conversion of ketone bodies to glucose could generate a significant gluconeogenic resource in the starved and diabetic states where fatty acid metabolism becomes elevated. Whilst the glyoxylate pathway, allowing the conversion of fatty acids to glucose tby by-passing the two CO_2 liberating steps of the Kreb's cycle, has long been recognised to exist in many plant and micro-organism species (Kornberg & Krebs, 1957) the presence of a gluconeogenic bridge between fatty acids and glucose has only more recently been established in mammalian systems.

Studies using ^{14}C acetone in humans showed that less than 30% of the labelled acetone was excreted in that form and the ^{14}C label appeared in glucose, carbon dioxide and amino-acids suggesting metabolism of acetone within the body (Reichard *et al.*, 1979). [2-^{14}C] labelled acetone was seen to be incorporated into blood glucose in diabetic ketotic humans (Owen *et al*, 1982). Similar studies in the post-mitochondrial fractions of rat livers showed that the labelled carbon atom, added into the system in acetone, was incorporated into lactate and if the liver fractions were prepared from starved animals the level of this encorporation was elevated (Coleman, 1980). Possible metabolic intermediates for the pathway converting acetone to glucose were suggested by the analysis of metabolites in the

blood of the people and animals used in these studies, principal amongst these was seen to be 1, 2-propandiol (Reichard *et al.*, 1979). Studies in rats began to dissect the possible gluconeogenic pathways for acetone. The initial oxidation of acetone to acetol was shown to be mediated by the microsomal fractions of rat livers and required the presence of oxygen and an NADPH regenerating system. The enzyme catalysing this step was termed the "acetone mono-oxygenase". Rats pre-treated with 1% acetone in their drinking water showed a ten-fold increase in their ability to catalyse this reaction. By addition of a glyoxylase trapping system, glutathione and glyoxylase I, to this reaction mixture D-lactate was seen to be produced. Similarly, pre-treatment of the rats with 1% acetone led to a four-fold increase in the ability of the microsomes to catalyse this reaction. Studies using mixtures of labelled and unlabelled putative intermediates in the conversion of acetone to lactate and glucose validated the proposed pathways; it was seen however that the pathway between methyl-glyoxal and D-lactate was not the principal route as addition of unlabelled D-lactate did not greatly reduce the level of labelled glucose produced after the addition of labelled acetone. 1, 2-propandiol, one of the intermediates of the acetone to glucose conversion identified in the blood, was mixed with the microsomal reaction leading to the generation of L-lactate. This reaction was blocked by methyl-pyrazole, an alcohol dehydrogenase inhibitor, and cyanamide, an aldehyde dehydrogenase inhibitor suggesting a role for these enzymes in the conversion of acetone to glucose. Perfusion studies suggested that the liver was not capable of generating all the 1, 2-propandiol present in the blood, suggesting the presence of an extrahepatic generating system metabolising acetol and returning it to the liver as the 1, 2-propandiol precursor for gluconeogenesis (Cassaza *et al.*, 1984). On the basis of this work two pathways for the gluconeogenic conversion of acetone were proposed to account for the conversion of acetone to pyruvate and so glucose. Firstly, the "methyl-glyoxal" pathway utilising acetol produced within the liver in conjunction with the acetone and acetol mono-oxygenase and glyoxylase enzymes. The second "propandiol" pathway used extrahepatically derived 1, 2-propandiol as its substrate (Cassaza *et al.*, 1984).

The nature of the acetone and acetol mono-oxygenase components of these systems, with their obvious similarities to P-450 in terms of the reactions catalysed and their requirements, were further investigated. The experiments in the rat were repeated in acetone treated and control rabbits. Attention was focussed on CYP2E as this enzyme had previously been shown to be induced by and metabolise, amongst many other small solvent molecules, acetone. The levels of activity and the levels by which acetone pre-treatment enhanced these activities were comparable to the results obtained in the rat. Antibodies to purified rabbit P-450 3a (CYP2E) blocked 90% of the acetone mono-oxygenase activity and 70% of the acetol mono-oxygenase components of the gluconeogenic pathway in acetone treated animals. In untreated animals, although the acetone mono-oxygenase

component was still blocked by 90%, the antibody to CYP2E only blocked 30% of the acetol mono-oxygenase activity suggesting that in the control state other P-450 may contribute to its metabolism. In reconstituted membrane assay systems using purified P-450 components CYP2B1, CYP2C3, and CYP1A2 were also shown to be capable of converting acetol to methyl-glyoxal suggesting potential roles for these P-450 as acetol mono-oxygenase. The relative activities of these isoforms in the catalysis of this reaction (nmol acetol hydroxylation/min/nmol P-450) were CYP2B1 2.48, CYP2E 12.00, CYP2C3 1.51, CYP1A2 0.55. CYP1A1 and CYP3A1 in the same reconstitution experiments were unable to catalyse the metabolism of acetol (Koop and Cassaza, 1986).

These results are summarised with additional enzymes which may potentially contribute to the pathways added in Figure 3.14. The fact that similar intermediates seen in the rabbit and rat proposed propandiol and methyl-glyoxal pathways are present in human suggest that these pathways may play a role in the metabolism of diabetes, starvation and fasting in this species also. It was noted in fasted humans that the rate of acetone metabolism was accelerated allowing up to 59% of the acetone produced to be metabolised. If this metabolised actone could potentially enter gluconeogenic pathways it would potentially contribute 11% of gluconeogenically produced glucose in 21 day fasted human subjects (Reichard *et al.*, 1979; Reichard *et al.,* 1986.). Other pathways for the metabolism of acetone, not passing through D- or L-lactate, have also been suggested based on the distribution of labelled carbon atoms obtained in glucose when animals are given specific carbon number labelled acetone (Kosugi *et al.*, 1986).

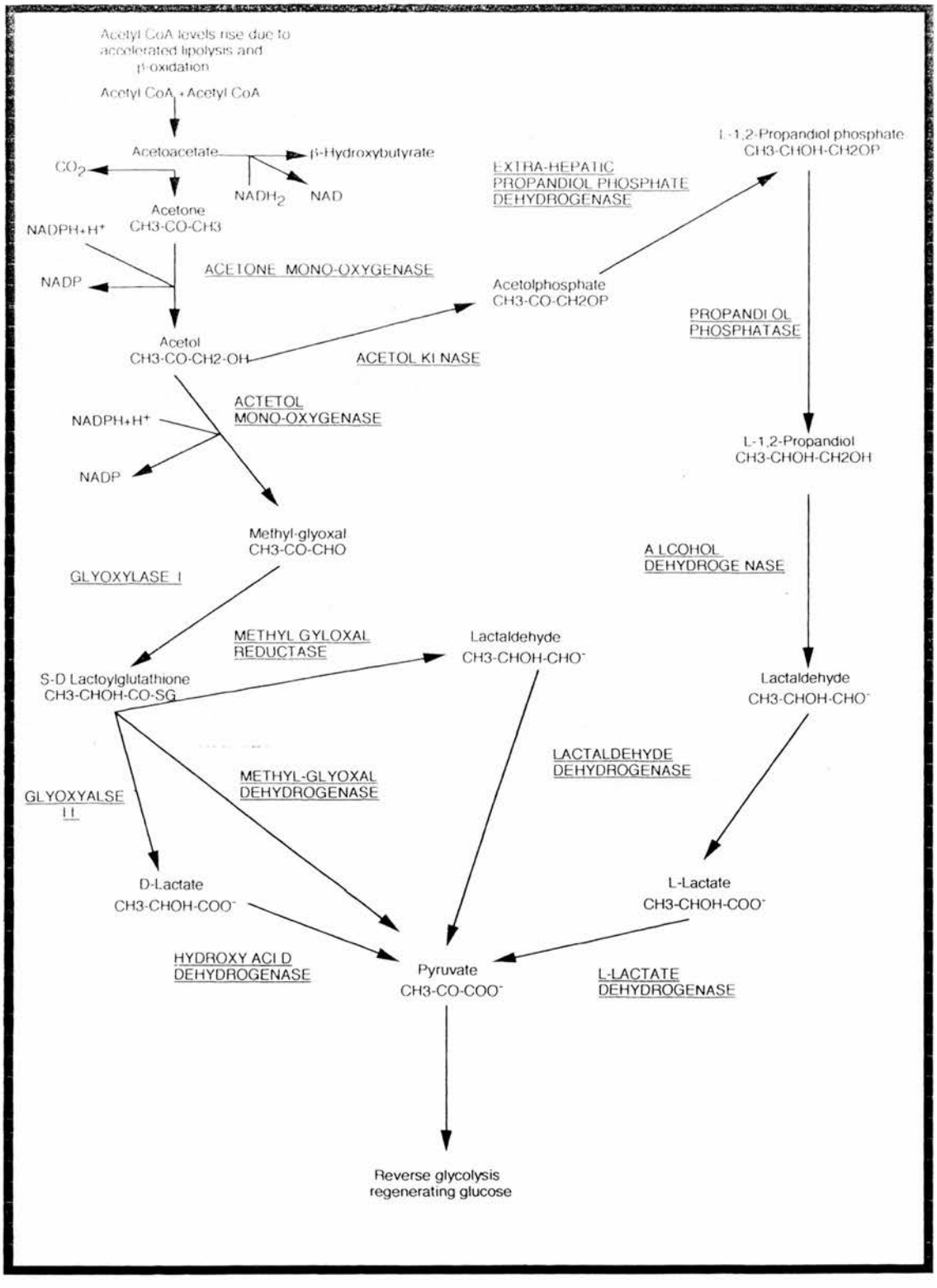


Figure 3.14: The proposed pathways for the gluconeogenic conversion of acetone to glucose involving the CYP2E subfamily. Acetone (top left), produced as a result of increased β-oxidation of fatty acids in diabetes and starvation, passes through the acetone-, and acetol-monoxygenase enzyme now known to be the CYP2E subfamily protein (**CYP2E**). The products of these reactions then pass through either the methyl-gloxal pathway (bottom left), or the propandiol (top right) gluconeogenic pathways to ultimately generate glucose. Figure compiled from information contained in Cassaza *et al.* (1984), Argiles (1986), and Koop and Cassaza (1986).

Section 3.10.2: An endogenous metabolic role for CYP2E as a rationale for the induction of this subfamily in diabetes and starvation

It is possible that CYP2E, with its capacity to act as acetol mono-oxygenase and as the major acetone mono-oxygenase, has a crucial role to play in intermediary metabolism acting to catalyse the gluconeogenic conversion of fatty acids to glucose via the bridging acetone molecule. As suggested by radiolabelling studies, these acetone conversion pathways may provide a sizeable quantity of gluconeogenically derived glucose in the starved or diabetic states (Reichard *et al.*, 1979). The induction of CYP2E, as proposed via the substrate stabilisation mediated action of acetone and an increase in the level of CYP2E mRNA, may thus represent a component of the body's adaptive response to starvation and diabetes. As a result of the CYP2E induction, acetone metabolism would be increased providing substrates for gluconeogenic pathways and the flux through the 1, 2-propandiol and methly-glyoxal gluconeogenic pathways would rise. In this manner the high levels of acetone produced, due to a switch to fatty acid metabolism in starvation and diabetes, could be converted into glucose. These pathways may also aid in the restoration of the pH balance of the blood in diabetes. The fact that the CYP2E response was seen at both the protein and mRNA levels in the kidney and that acetone has been seen to induce the mouse Cyp2e1 protein in a variety of extra-hepatic tissues (Section 4.4.2) suggests that it may provide acetol to extrahepatic 1,2-propandiol generating systems throughout the body.

Section 3.10.3: A role for the CYP4A subfamily in fatty acid metabolism in diabetes

Peroxisomal proliferators induce the CYP4A subfamily transcriptionally and the physiological signalling pathways involved in this action, potentially activated by increases in circulating free-fatty acids, may be the same as those inducing CYP4A in diabetes. Other genes activated by peroxisome proliferators, seemingly in a co-ordinate manner, are associated with the generation of an increase in the level of cyanide-insensitive β-oxidation within the peroxisomes. These genes include most of the components of the peroxisome associated β-oxidation pathway, the peroxisomal fatty acyl-CoA oxidase, the enoyl-CoA hydratase/3-Hydroxyacyl-CoA dehydrogenase and both the carnitine and palmitoyl transferases; other components of the peroxisome, such as catalase, remain unaffected suggesting a specific and co-ordinate induction only of those components associated with fatty acid metabolism (Reddy *et al.*, 1986). Results gained from this and other studies (Hoire *et al.*, 1981) suggest that diabetes leads to an activation of peroxisomal β-oxidation, potentially progressing to peroxisomal proliferation in extreme cases. It is possible therefore that the induction of the CYP4A subfamily by both peroxisomal proliferators and

diabetes also reflects the shift from the use of glucose to the use of free-fatty acids as the fuel source in these states.

The CYP4A subfamily members have been shown to metabolise medium chain fatty acids by ω and ω-1 hydroxylation; the ω-hydroxylation reaction is however the reaction predominantly catalysed by the CYP4A subfamily (CaJacob *et al.*, 1988). The primary alcohols produced by these reactions can then be converted to dicarboxylic acids within the body by aldehyde and alcohol dehydrogenases. The presence of such medium chain dicarboxylic acids has been noted in the urine of rats and humans and it was seen that the levels of these compounds were dramatically elevated in fasted, high fat content fed and diabetic animals; by using [1-^{14}C] labelled medium chain fatty acids the rate of ω–hydroxylation was seen to increase in these states (Mortensen & Gregersen, 1981). As [1-^{14}C] labelled medium chain fatty acids were used then direct β-oxidation would lead to a loss of the label in the first acyl-CoA generated as the thiol esterification reaction with CoA would occur only with the labelled carboxylic acid group. Labelled dicarboxylic acids however were recovered suggesting that ω-hydroxylation, and so generation of a carboxy group at both ends of the molecule, occurred prior to β-oxidation (Mortensen & Gregersen, 1981). By inducing peroxisomes in rats by treatment with clofibric acid and then blocking mitochondrial β-oxidation with cyanide it was seen that these dicarboxylic acid molecules were actively consumed by peroxisomal β-oxidation (Mortensen *et al.*, 1982).

These observations suggest that in the diabetic state the body increases the release of free-fatty acids and accelerates β-oxidation, the levels of CYP4A are also increased and this induction manifests itself on intermediary metabolism through an increase in the level of production of dicarboxylic fatty acids. These dicarboxylic acids can, as well as the more normal mono-carboxylic fatty acids, enter into peroxisomal β-oxidation. It is of interest in the proposed co-ordinate nature of CYP4A and peroxisomal β-oxidation that genetic defects in mitochondrial β-oxidation, for example in the mitochondrial acyl-CoA dehydrogenase, or in valproate treatment which inhibits the mitochondrial acyl-CoA dehydrogenase that the levels of urinary dicarboxylic acids also become elevated (Gregersen *et al.*, 1976). In these instances it is possible that, as mitochondrial β-oxidation fails, situations in which lipolysis occurs lead to a more rapid rise in circulating free-fatty acids and the induction of peroxisomal β-oxidation and CYP4A as suggested by the presence of the dicarboxylic acids. The fact that a possible co-ordinate activation of CYP4A and the components of the peroxisomal β-oxidation components occurs suggests that an endogenous role for the CYP4A subfamily may lie in their capacity to ω-hydroxylate medium chain free-fatty acids.

A role for the CYP4A subfamily induction in diabetes in the acceleration of ω-hydroxylation of fatty acids

In the past the process of ω–hydroxylation of fatty acids, and the presence of dicarboxylic acids and their oxidation by both the mitochondrial and peroxisomal β-oxidation pathways, even though it was seen to be prevalent particularly in the starved state, has been viewed essentially as a biochemical quirk. Early investigations into the amount of free-fatty acids that become ω-hydroxylated suggested that a high proportion, around 20% of laurate and 10% of palmitate, underwent ω-hydroxylation in starved animals, in the control animals the levels were very low (Kam *et al.*, 1978). Potentially what this process allows however is very pertinent to the needs of the body in a starved and diabetic state, those states in which ω–hydroxylation and di-carboxylic acid production have previously been noted to become elevated.
ω-Hydroxylation of an even carbon numbered free-fatty acid, followed by complete oxidation of the even-carbon dicarboxylic fatty acid produced, generates succinyl-CoA, a precursor of succinate. Succinate, on entering the Kreb's cycle, could then lead to the generation of pyruvate and so, by reverse glycolysis, glucose. Free-fatty acid labelling studies have shown that this process occurs *in vitro* in both hepatic and extrahepatic tissues (Kolvaa & Gregersen, 1986; Vameq & Draye, 1989). CYP4A may therefore represent a component within a gluconeogenic pathway allowing a link between the accelerated level of fatty acid metabolism in the starved and diabetic states and gluconeogenesis.

Section 3.10.4: Possible endogenous roles for the other P-450 seen to be induced in diabetes

The proposed gluconeogenic endogenous roles for the CYP2E subfamily, via acetone metabolism, and the CYP4A subfamily, via dicarboxylic acid generation, may also be the rationale behind the induction of the other P-450 seen in the diabetic state. The CYP1A and CYP2B subfamily were seen to be capable of acting as acetol-monooxygenase components in reconstitution investigations into the methyl-glyoxal pathway for acetone gluconeogenesis although with a much lower level of activity than CYP2E; CYP3A however was not seen to be capable of catalysing this reaction (Koop and Cassaza, 1986). It is possible that the increase in CYP3A levels, and those members of the CYP2C subfamily involved in steroid biotransformation seen to be elevated (Yamazoe *et al.*, 1989; Favreau *et al.*,1987a), may relate to a possible role in increased, possibly degradative, metabolism of the elevated levels of steroids and cholesterol present in the circulation in diabetes. It is possible that the products generated from the steroids metabolised by these P-450 may themselves have a biological function; this has been seen to be the case in some

of the metabolites of testosterone generated by the catalytic activities of CYP2C subfamily for example (Sonderfan & Parkinson, 1988).

Section 3.11: Changes in other components of the xenobiotic metabolising system in diabetes

As well as changes in the levels of certain P-450 the effect of the diabetic state on some components of phase II, associated with the removal of reactive metabolites and xenobiotics, was investigated. The possible effect which diabetes produces on glyoxylase I, in connection with its proposed role in the methly-glyoxal gluconeogenic pathway (Section 3.10.1), was investigated. The effect of the diabetic state on the levels of certain glutathione S-transferases (GSTs), as well as its general effect on the glutathione conjugation activity were investigated in order to gauge the potential ability to conjugate activated xenobiotic potentially generated as a result of the inductions of the P-450 seen. In this context examples of increases in the activties of *N*-acetylases, an enzyme activity associated with phase II metabolism of xenobiotics, with the onset of diabetes in the BB/E rat has previously been reported (Lindsay & Baird, 1990).

Section 3.11.1: Glyoxylase I and the diabetic state

The glyoxylase system has been proposed to play a role in the methyl-glyoxal pathway of acetone gluconeogenesis (Cassaza *et al.*, 1984). The effect of type I diabetes on the activity of the glyoxylate cycle in humans was studied in red blood cells and an increase in the activity of gloxylase I was noted (Thornalley, 1990). The use of anuclear red blood cells however may not allow the assessment of changes in glyoxylase enzyme activities possibly resulting from transcriptional changes. To investigate this possibility the effect of diabetes on the level of glyoxalase I was studied in the cytoplasm of male rats using a polyclonal antibody to purified mouse glyoxylase I (kind gift, Dr L. McClellen, Department of Clinical Biochemistry, The Royal Infirmary, Edinburgh.). Diabetes however was seen not to change the protein level of glyoxylase I (Figure 3. 15). The increased glyoxylase I activity previously seen (Thornalley, 1990) may potentially relate to a post-translational change in the pre-existing enzyme. It has been seen that both glycerol and 12-O-tetradecanoylphorbol-13-acetate (TPA) activate glyoxylase I in leukocytes; TPA may mediate the changes in glyoxylase activity via phosphorylational changes in the enzyme (Gillespie. , 1981). It is interesting that glycerol has the ability to activate glyoxylase I; this feature could allow an increase in the flux through the methyl-glyoxal pathway whenglycerol levels become elevated due to the activation of lipolysis in diabetes, fasting, and starvation.

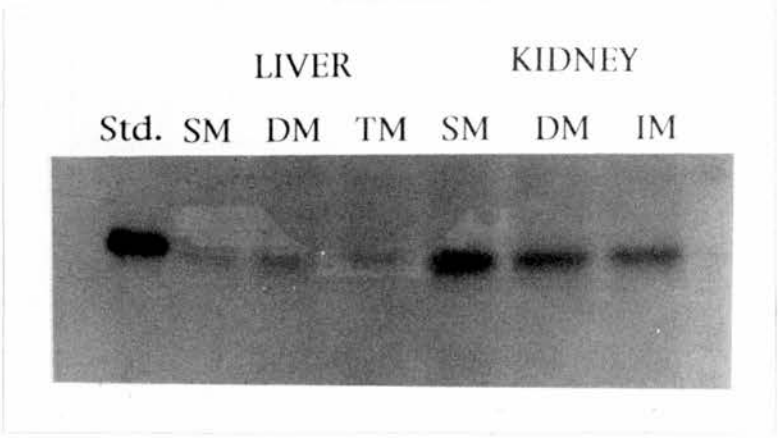


Figure 3.15: The effect of diabetes on the protein level of glyoxylase I in the male BB/E rat. Total cytosolic protein (10μg of liver and 20μg of kidney) isolated from BB/E subline male (**SM**), diabetes-detected, diabetes-prone male (**DM**), and diabetes-detected, insulin-treated, diabetes-prone male (**IM**) were separated by SDS/PAGE on 12% gels. The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies raised to purified mouse gloxylase I protein. No bands other than those shown were detected. The standard (**Std.**) was prepared from glyoxylase I purified from mouse liver cytosols and the glyoxylase I protein ran with a M_r of approximately 22,000.

Section 3.11.2: Modifications in GST levels and activities in diabetes

In viewing P-450 classes 1 to 3 as xenobiotic metabolising enzymes the effects of the potentially injurious reactive products which they can generate can be diminished by their conjugation with cofactors, such as glutathione, allowing subsequent excretion (Section 1.1). Situations are encountered where xenobiotics, such as phenobarbital and 3-methylcholanthrene generate a concomitant increase in both the levels of P-450 and GST thus potentially allowing the P-450 generated activation and GST mediated conjugation steps to potentially co-ordinately accelerate the removal of xenobiotics (Pickett *et al.*, 1984). In view of the increases in the levels of P-450 in diabetes the effect which this state, and so in xenobiotic metabolising terms the level of protection available against reactive metabolites, on GST levels of was investigated. As shown by this study diabetes produced no change in the level, either protein or mRNA, of the microsomal GST; the microsomal GST however is genetically and evolutionarily distinct from the cytosolic classes of GST (Ketterer *et al.*, 1988).

Previous activity based assessments on the effect of diabetes in chemically induced rats and mice on the level of GST activity, using the conjugation of glutathione to 1-chloro-2,4-dinitrobenzene (CDNB) as a marker for total GST activity, have produced conflicting results. One study using alloxan and streptozotocin-treated rats demonstrated a short-term increase in the level of CDNB conjugation with diabetes. The increase in CDNB activity returned to control levels after one week and below control levels after two weeks. Insulin treatment of the diabetic animals with depressed GST activity generated an increase in CDNB activity above the activity of the control animals (Carnovale *et al.*, 1990). In a study of streptozotocin-treated mice an increase in the level of GST activity was reported to occur for several weeks during diabetes (Agius & Gidani, 1985). Long-term decrease in the levels of activity of GSTs was noted in other studies however (Watkins *et al.*, 1988; Grant & Duthie, 1987; Aniya *et al.*, 1989). The variability in the results obtained suggests that they may reflect the degree of severity of the disorder produced as the times which the animals were left in a diabetic state without insulin therapy were very long.

Western blot analysis of three classes of rat GST in male liver cytosols of the BB/E rats used in this study revealed that the diabetic state did not produce any profound effects, either inductive or repressive, on the protein levels of any of the GSTs examined (Figure 3.16). CDNB assays of the cytosols however suggest that the level of GST activity is increased in the diabetic state and treatment of the animals with insulin generates a further rise in the general GST conjugation ability of the cytosols (Table 3.4).

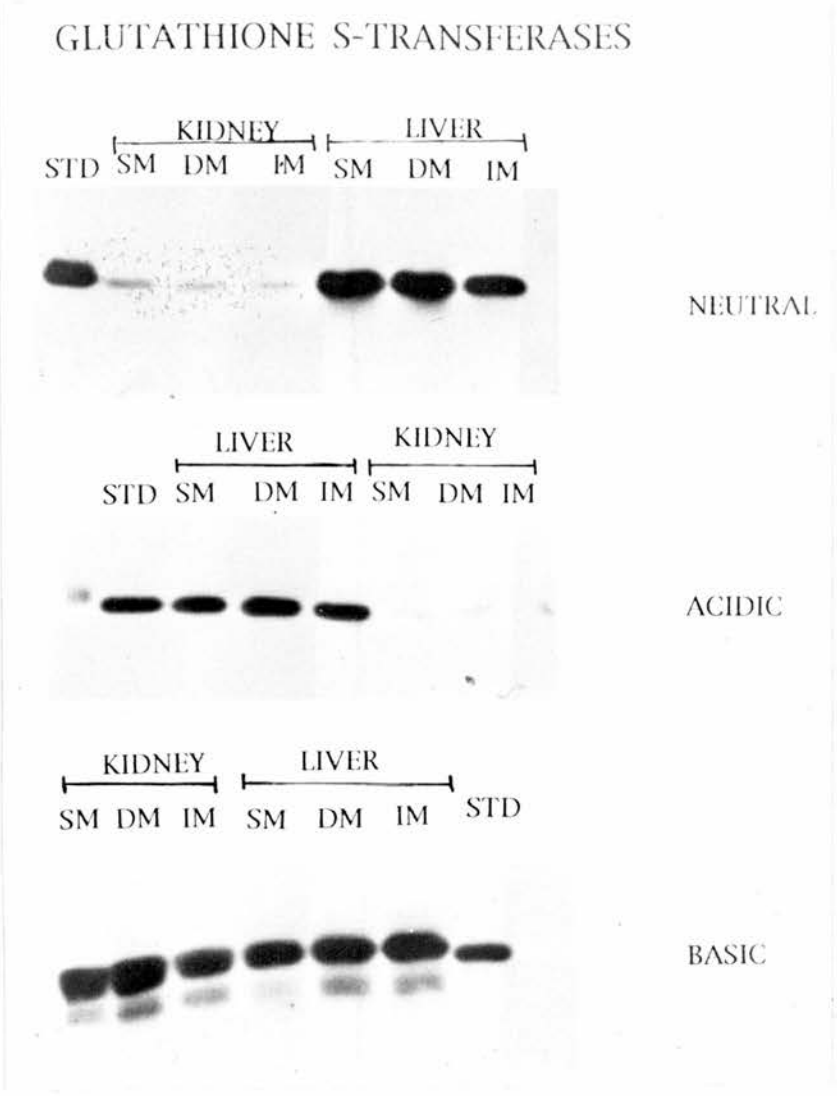


Figure 3.16: Western blot analysis of the effect of diabetes on the protein level of certain GST enzymes in the male BB/E rat. Total cytosolic protein (10μg of liver and 20μg of kidney) isolated from BB/E subline male (**SM**), diabetes-detected, diabetes-prone male (**DM**), and diabetes-detected, insulin-treated, diabetes-prone male (**IM**) were separated by SDS/PAGE on 12% gels.The proteins were transferred to nitrocellulose filters and probed using polyclonal antibodies raised to various purified rat GST enzymes as indicated. No bands other than those shown were detected. The standards (**STD**) were prepared from rat liver cytosols and the GST proteins all ran with a M_r of approximately 23,000.

This increased CDNB activity, without a rise in the level of the GST proteins analysed, may be accounted for by the possibility that other classes of GST not recognised by the polyclonal antibodies employed may become elevated as there are at least ten subunits capable of this activity in the rat (Ketterer *et al.*, 1988) It is also possible that the kinetic parameters of the pre-existing enzymes may be modified in a similar manner to that proposed for the glyoxylase I enzyme (Gillespie, 1981) and reports have suggested the possible role of both phosphorylational and methylational modifications in mediating such events (Siegel, *et al.* , 1990). The total CDNB assay results however suggest that diabetes may produce a rise in the ability to generate glutathione conjugates and this rise may reflect an increased xenobiotic metabolising ability due to the elevated P-450 levels.

Table 3.4: The effect which the diabetic state produced on the ability of rat liver cytosols to catalyse the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) was investigated. All assays were performed in triplicate; the assays were performed in collaboration with Dr. L. I. McClellan, Department of Clinical Biochemistry, The Royal Infirmary, Edinburgh.

	Glutathione conjugation of CDNB, nmol per min per mg cytosol protein	
	x	σn-1
SUBLINE MALE	698.02	(20.21)
SUBLINE FEMALE	626.03	(6.28)
DIABETIC MALE	776.23	(11.44)
DIABETIC FEMALE	910.89 859.75	(20.39) (14.11)
INSULIN TREATED MALE	1841.33	(18.77)
INSULIN TREATED FEMALE	1219.39	(10.64)

Section 3.12: Xenobiotic metabolism and chemical carcinogenesis in diabetes; the implications of increased P-450 levels

Studies on the effect of diabetes on the metabolism and activation of a variety of compounds indicate that these processes are accelerated in chemically-induced diabetic animals. The hepatotoxicity of carbon-tetrachloride (Hanasono *et al.*, 1975a and b), chloroform (Aniya *et al.*, 1989), thioacetamide (El-Hawari & Plaa, 1983), and certain

nitrosamines (Lorr *et al.*, 1984) were all seen to be elevated in the diabetic state for example. As suggested in this study endogenous induction of P-450, to perform potential gluconeogenic roles, manifest themselves in an increased level of xenobiotic metabolism and resultant toxicity; in a similar manner the non-genotoxic effects associated with damage produced in situations of accelerated peroxisomal activity may also be expected to increase (Reddy *et al.*, 1992). In diabetic humans, if similar endogenous roles are performed by certain P-450, in periods of poor metabolic control where levels of insulin fall and acute periods of hypoglycaemia and lipidaemia are experienced, the endogenous induction of certain P-450 would be predicted to occur. This has been seen to be the case for the CYP2E subfamily which is seen to be elevated in the lymphocytes of diabetic subjects (Song *et al.*, 1990). The induction of these P-450 would result potentially in an increase in the capacity to metabolise and activate xenobiotics. Similar situations would also be predicted to arise as the result of periods of fasting and starvation where again metabolic changes would generate signals leading to the increase in certain P-450 levels.

Could such periods of heightened xenobiotic sensitivity lead to, as has been seen in animal models, increases in the levels of their toxicity and even carcinogenicity? In this context it is of note that the diabetic BB rat has a marked increase in the incidence of certain cancers far above the levels in the diabetes-resistant subline. The long-term insulin maintained diabetic animals show around a 15% incidence of lymphomas as opposed a 1% incidence in the non-diabetic diabetes resistant subline for example (Kalant & Seemayer, 1979; Seemayer *et al.*, 1982; Seemayer *et al.*, 1983). Could the higher incidence of hyperlipidaemia and thus potential periods of higher vulnerability to xenobiotics result in higher cancer incidence in human diabetic subjects ?

A major survey looking at cancer mortality in human diabetic subjects was undertaken over a twenty-six year period looking at a cohort of 21,447 people. The numbers of specific cancers and the overall level of cancers leading to mortality was compared against the expected mortality rates in the non-diabetic population. This report indicated that there appeared to be no change in the overall level of cancer mortality amongst female diabetic subjects and that the level of cancer mortality in male diabetic subjects actually is reduced by around 15%. The reduction in male cancer mortality rate is however offset by the affects of diabetes-associated complications such as hypertension and coronary heart disease (Kessler, 1970). In general diabetic subjects have, due to the complications associated with the disorder, an approximately 30% reduced life-expectancy as compared non-diabetics (Green & Hougard, 1984). In the context of the potential effect of periods of P-450 induction on xenobiotic activation therefore the effect of the diabetic state on the incidence of specific cancers, rather than the mortality generated by them, would be more illuminating.

Section 3.13: Roles for the changes in P-450 in the aetiology of diabetes

The causes of type I insulin-dependent diabetes are not known. In human, rat and mouse, diabetes appears to be an autoimmune disease in which the β-cells, comprising less than 2% of the total pancreatic mass are destroyed. Circulating antibodies to several β-cell surface antigens and insulin are seen to be present in the blood; immunisation with insulin itself, however, does not lead to insulitis and β-cell destruction (Dean *et al.*, 1987; Zeigler *et al.*, 1989). The initial events leading to autoimmunity are however obscure and, although genes associated with the functioning of the immune system have been linked to the susceptibility of developing the disorder, the development is clearly multi-factorial (Todd *et al.*, 1987). Clearly environmental factors play a large role in the initiation of the disorder as illustrated by monozygotic twin studies on human diabetics showing a 50% discordance in the development of the disorder (Leslie & Pyke, 1980). Whether the autoimmune aspect of the disorder correlates with differences in the immune system allowing the β-cell destruction, or whether other events lead initially to a release of antigens previously unseen from the β-cell triggering this auto-immune response is unclear. The β-cells are highly susceptible to oxidative damage as demonstrated by their rapid destruction by alloxan and streptozotocin and subsequent generation of insulitis (Section 3.2.1). These observations make an initial environmental input into β-cell destruction, leading to antigen release, in association potentially with a predisposition in the immune system to generate auto-immune responses, a possibility in the initiation of the disorder (Malaisse *et al.*, 1982; Assayama *et al.*, 1986; Nomikos *et al.*, 1986). If such a scenario could be envisaged then, in association with genetic predisposition to generate an auto-immune response, the triggering event may be environmental. No single environmental factor alone need be solely responsible and multifactorial events would present the same phenotype due to the fragility of the β-cells.

Several environmental factors have been suggested to be capable of triggering this initiation event. Viral infection has been associated with β-cell destruction; Epstein-Barr, Coxsackie B4 and congenital rubella infection have all been associated with β–cell destruction however it is not clear if the β–cell infection by these agents has preceded their destruction or followed it. In the case of Rubella infection it is of note that a predisposition to other auto-immune diseases, such as thyroiditis, is seen potentially taking the place of the genetic predisposition associated with the immune-system (Helmke *et al.*, 1986. Tuverno *et al.*, 1988). Other factors leading to a triggering of the disorder may relate to xenobiotics both in the environment and in the diet illustrated by the use of alloxan and streptozotocin in the establishment of animal models of the disorder.

Some studies have indicated potential environmental chemicals which may participate in the initiation of the destrucyion of the β-cells. The rodenticide "Vacor", with an *N*-3-pyridyl

methyl-*N*-*p*-nitrophenylurea active component, produced similar effects in the β-cells of humans accidentally exposed to it illustrating the common theme of β–cell fragility in the development of the disorder in humans also (Lee *et al.*, 1977). The potential triggering of β–cell destruction by xenobiotics in foodstuffs has been noted. A relationship between parental consumption of smoked cured mutton, seasonally consumed in Denmark during December, and the appearance of a high incidence of diabetes of children born in October was seen (Berne *et al.*, 1974). This observation was further investigated using mice fed similar nitrosamine rich foods at various stages prior to and during pregnancy. Diabetes resulted in 16% of male and 5% of female offspring as a result of these treatments against a very low control incidence suggesting that environmental nitrosamines may be triggering β–cell damage *in utero* (Helgason *et al.*, 1982). It is of note in the context of the potential role of nitrosamines in the triggering of β–cell destruction that streptozotocin, a strongly diabetogenic compound produced by the soil fungus *Streptomyces achromogenes*, is one of the few naturally occuring nitrosamines (Herr *et al.*, 1967).

Although certain xenobiotics seem to be capable of triggering β–cell destruction on their own in a more general sense it seems probable that a wide variety of factors both genetic and environment, contribute to the potential development of the disorder. It is particularly interesting, in the light of the observation that metabolic changes in diabetes induce P-450 levels, that some of the xenobiotics implicated in the triggering of β–cell destruction can potentially be metabolised and activated by P-450.

Another interesting observation is the suggested involvement of radicals in the further reactions of glycated proteins to generate advanced glycosylation, or AGE products (Hunt *et al.*, 1988; Wolff *et al.*, 1989). These products are associated with the complications of the metabolic disorder, leading to cataracts, kidney failure, neuropathy, joint stiffening, hypertension and cardiac failure (Section 3.2). P-450 enzymes have been seen to generate free radicals and have been implicated in the past in the generation of lipid peroxidation as a result of this action (Gorsky *et al.*, 1984). It is an intriguing possibility that the elevation of P-450 enzymes in diabetes, and so an elevation in their radical generating activities, may contribute to the generation of increased AGE products and so the long-term side effects associated with diabetes.

Section 3.14: P-450 expression and induction in the pancreas; implications for the destruction of the β–cell in diabetes and possible roles in these cells

The presence of Cyp2e1 in the pancreas of mice and its induction by acetone was noted in Western blot analysis (Section 4.4.2, Figure 4.3). The CYP4A subfamily was seen to be expressed in the BB/E rat pancreas by imunohistochemical analysis and more specifically in the insulin producing β-cells (Figure 3.17).

igure 3.17: Immunohistochemical localisation of the CYP4A subfamily to the β-
ells of the pancreatic islet of the BB/E rat. The structure of the pancreas was
etermined by haematoxylin and eosin (H/E) staining seen in the upper half of the
gures.The presence of the CYP4A subfamily proteins in the β-cells of the pancreatic
let was determined using polyclonal antisera raised to purified rat CYP4A subfamily
roteins. The antibodies recognising the CYP4A protein were complexed with
otinylated swine anti-rabbit antisera and the immune-complex detected using an
vidin-biotin peroxidase detection system with its substrate, 3, 3-diaminobenzidine,
ving the brown staining seen in the lower half of the figures. **A:** The pancreas of a
abetes-resistant, subline male BB/E rat showing normal pancreatic islets on staining
ith H/E and weak staining with the CYP4A antisera. **B:** The pancreas of a diabetes-
etected, diabetes-prone male BB/E rat showing insulitis in the pancreatic islets marked
/ infiltration with mononuclear imflammatory cells on H/E staining. The β-cells show
ear expression of the CYP4A subfamily proteins on staining with the CYP4A
ntisera. This analysis was performed in collaboration with Dr D. J. Harrison and Mrs
. Wolf, Department of Pathology, Edinburgh University.

A.

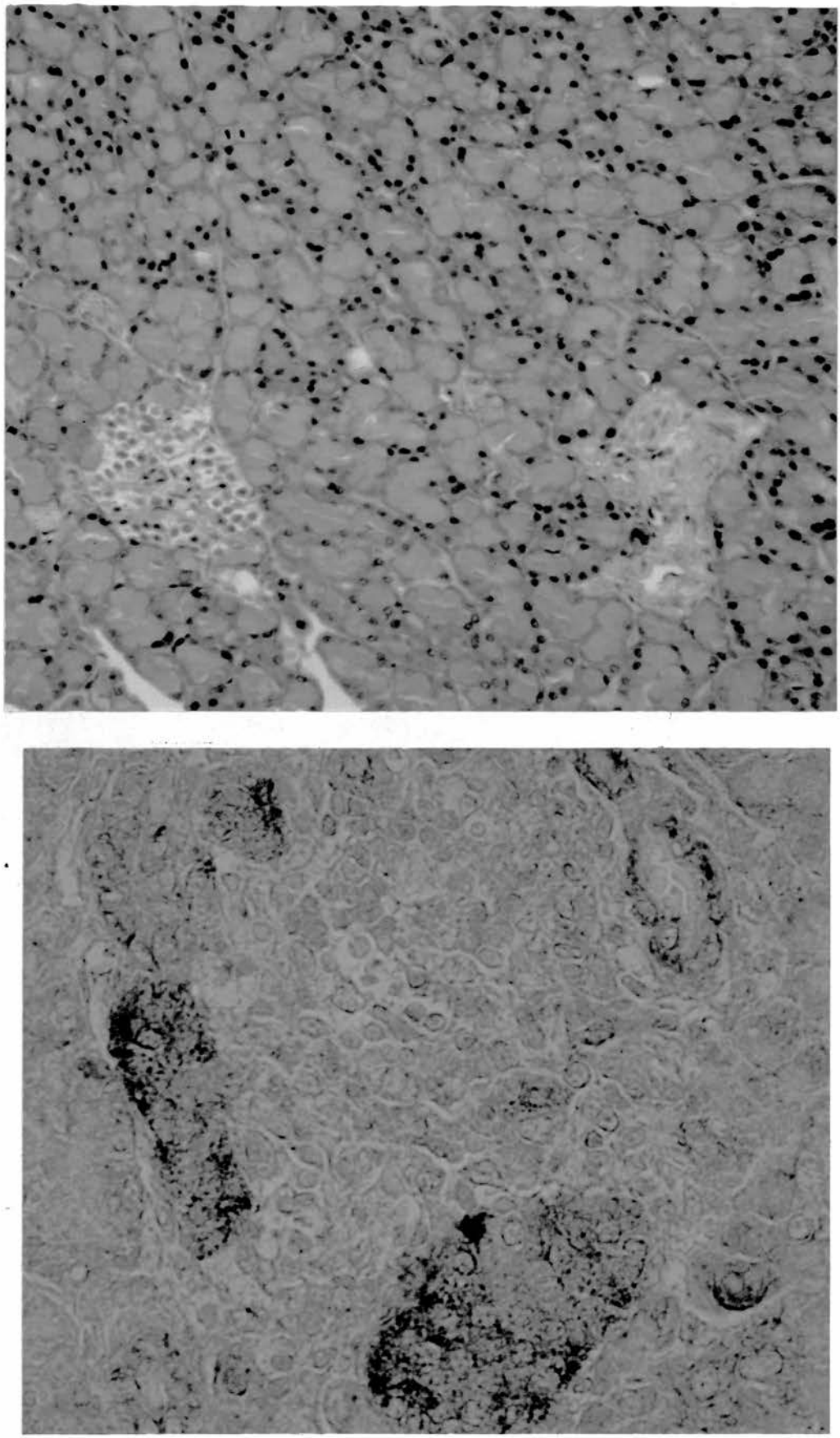


Figure 3.17 (Continued).

B.

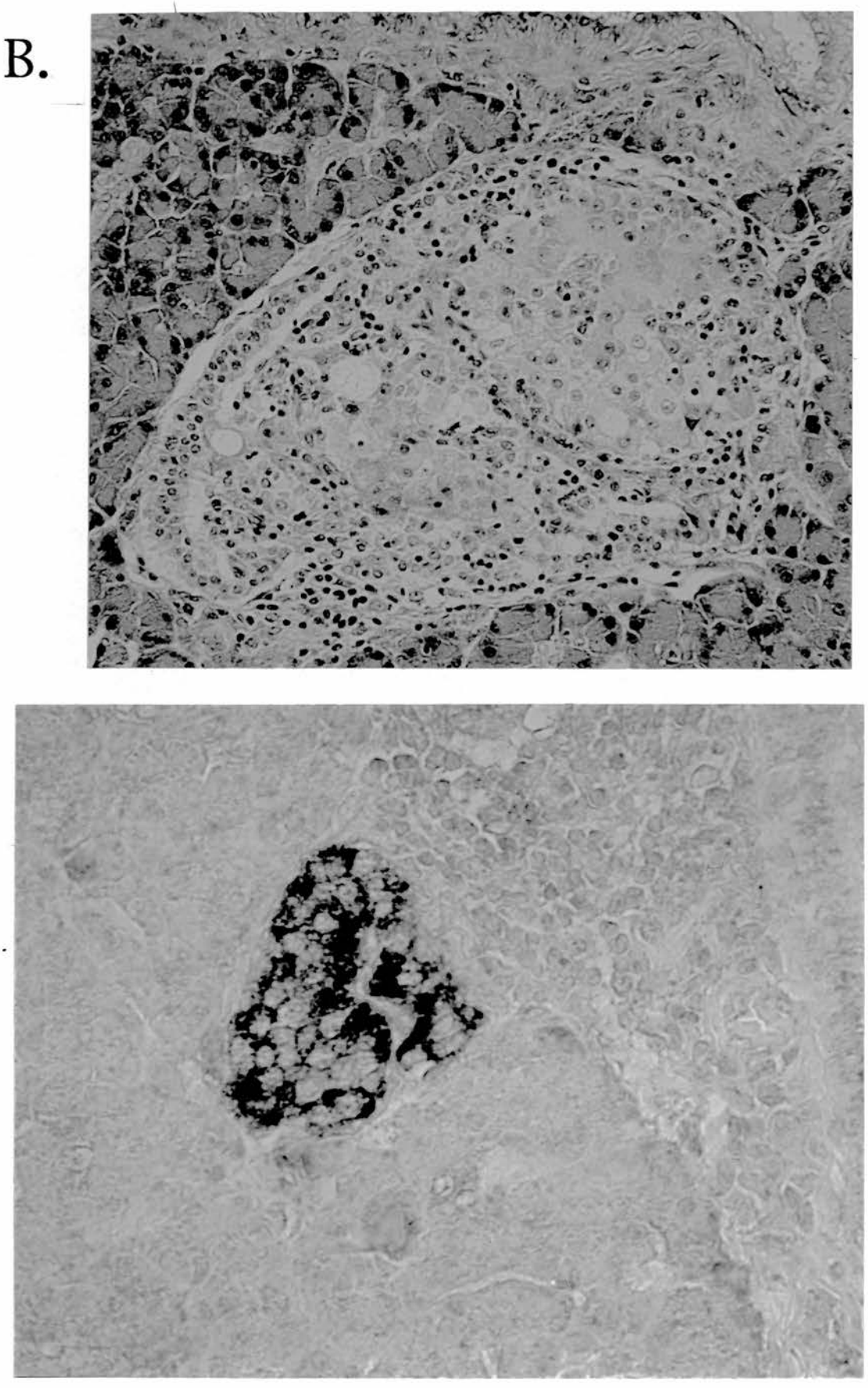


Figure 3.17 (Continued).

Although only weakly detectable in the β-cells in the control BB/E animals the level of CYP4A protein in the β-cells was seen to rise markedly with the onset of diabetes (Figure 3.17). The presence of P-450 in the pancreas suggests that they may play a role in the activation of xenobiotics within that organ; the potential generation of reactive metabolites by P-450 in the pancreas could contribute to β–cell DNA and membrane permeability damage either directly, through increased levels of lipid peroxidation, or indirectly through the generation of free-radicals. If endogenous controls led to an induction of P-450 in the pancreas, as they appear to do for Cyp2e on acetone treatment (Section 4.4.2) and the CYP4A subfamily (Figure 3.17), an initial β–cell damaging event, be it viral, genetic or environmental, leading to a fall in the levels of insulin would, by switching metabolism to the use of fatty acids as a fuel and so inducing P450, accelerate the damaging effect of P450 action in the pancreas. If a diabetogenic xenobiotic was principally activated by P-450 then the actual triggering event of β–cell destruction could potentially be mediated by the presence of the P-450 within the β–cell.

Given the potential damaging effect which activated compounds and radicals can produce on the β–cell what potential role could P-450 be playing in their presence in this cell type? The mechanisms controlling the release of insulin within β-cells is poorly understood and is highly complex involving cross-talk between other endocrine and paracrine signalling pathways and metabolite levels. Together these signals must allow careful monitoring of the blood and tissue levels of various fuels and respond to changes in these fuels by generating signals leading to the co-ordinating of the various enzymes involved in cellular energy metabolism. The mechanism by which the body can monitor the levels of such metabolites is poorly understood.

The β-cells are sensitive to the levels of glucose and controlling signalling proteins such as glucagon-like protein-1 (GLP-1) for example. GLP-1 in the presence of glucose generates elevations in intracellular cAMP and calcium levels and controls the permeability of potassium channels in the β-cell membrane. Via these signalling pathways the ambient levels of glucose determine the rate of pro-insulin production and insulin secretion (Holtz *et al.*, 1993).

Another signalling system that has been reported to allow secretory responses within the pancreas is through the generation of the biologically active metabolites of arachidonic acid, certain of these components such as the cis-epoxyeicosatrienoic acids (EETs) have been shown to generate insulin release from isolated β-cells for example (Falck *et al.*, 1983). Arachidonic acid is metabolised by three main pathways to generate the products which are active secondary messengers within cells. Arachidonic acid is metabolised by cyclo-oxygenase and lipo-oxygenase pathways to generate biologically active eicosanoids and a mono-oxygenase pathway generating the cis-epoxyeicosatrienoic acids (EETs), hydroxy-eicosatrienoic acids (HETEs), and ω– and ω-1 biologically active forms of arachidonic acid

(Needleman *et al.*, 1986). The mono-oxygenase component of arachidonic acid metabolism is performed by cytochrome P-450 (Fitzpatrick & Murphy, 1989). The EET and HETE signalling molecules have been demonstrated to generate stimulus coupling in endocrine, renal, ocular and secretory cells and as mentioned specifically in this context generate insulin release from the β-cells. These signalling compounds appear to mediate their actions via modulations in intracellular calcium possibly in a similar manner to the better understood action of the phosphatidyl-inositol signalling pathway (Kutsey *et al.*, 1983). Hormones such as vasopressin have been shown to elevate the levels of EETs and HETEs within a cell leading to release of intracellular calcium stores and these effects are abolished by the addition of P-450 inhibitors such as SKF-525A and ketoconazole (Holm *et al.*, 1989). The specific P-450 involved in the generation of the biologically active arachidonic acid are unclear although inhibitory antibody studies suggest a role for the CYP1A family (Holm *et al.*, 1989) and the presence of ω and ω-1 hydroxylation reactions suggests a possible role for CYP4A in these processes.

Given the presence of Cyp2e and CYP4A in the pancreas and more specifically of CYP4A in the β–cell itself and the ability of EETs to elicit a secretory response from β-cells (Falk *et al.*, 1983.) the possibility that certain P-450 in the β–cell may act as sensors to the prevailing fatty acid levels and respond to these through a signalling mechanism employing HETEs and EETs is intriguing. These P-450, seen to be induced in diabetes, could respond to ambiant fatty acid levels through endogenous induction pathways and, through the changes in their metabolic activities, generate signals reflecting the ambient levels of fatty acids. Possible roles of P-450 in signalling processes within the cell are a matter of speculation but are clearly highly intriguing (Nebert, 1990). These signalling molecules could well represent the ligands controlling the large numbers of "orphan" steroid hormone receptor superfamily proteins suggested to be involved in intracrine cellular signalling (O' Malley, 1990). The observed low affinity of peroxisome proliferators for the PPAR (Isseman & Green, 1990; Gottlicher *et al.*, 1992), and also the low afinity of other xenobiotics, such as phenobarbital, for other P450 associated transcription factors (Okey, 1990), could be reconciled if the actions of the xenobiotic were mediated indirectly through paracrine signalling systems. It is interesting in the context of a role for these signalling systems in diabetes that vasopressin, in stimulating the elevation of HETEs and EETs leading to the modulation of intracellular calcium levels, generates the activation of phosphorylase-a and so the activation of glycogenolysis in hepatocytes suggesting that these signalling systems may be involved in the regulation of intracellular energy metabolism (Yoshida *et al.*, 1990).

Section 3.15: Summary: P-450 Subfamilies 1 to 4; enzymes with endogenous roles marked by xenobiotic metabolism

It appears that certain members of the P-450 1 to 4 families possess endogenous roles in the diabetic state and are controlled by endogenous signalling systems. By analogy, physiological states such as fasting, where similar but less exaggerated metabolic and hormonal perturbations occur, will generate similar signals producing similar P-450 elevations. Such elevations have been seen to occur as would be expected from this postulate in starvation in the CYP2B and CYP2E subfamilies in the rat (Johansson *et al.*, 1988), and in the Cyp2e1 subfamily in the mouse (Section 4.5). The increases in P-450 levels manifest themelves in diabetes through an increased capacity to metabolise xenobiotics (Hanasono *et al.*, 1975 a and b; Aniya *et al.*, 1989; El-Hawri *et al.* , 1983; Lorr *et al.*, 1984).

Some of these enzymes, for example the CYP2E and CYP4A subfamilies, possess potential roles in the acceleration of gluconeogenic return of the byproducts of increased fatty acid metabolism; P-450 increases, through their contribution to the mono-oxidase metabolism of arachidonic acid, may also potentially act as sensors for ambient fatty acid levels in the body.

One interpretation of these observations is that there is an endogenous role for these P-450 families in intermediary metabolism; this role however has been overlooked as a result of the ability of these enzymes to activate xenobiotics and so contribute to carcinogenesis. An alternative interpretation, however, might be that the potential roles of these enzymes in gluconeogenesis and intracellular signalling are now replaced by their roles as catalysts of xenobiotic metabolism. The roles in intermediary metabolism, it would be argued, have now essentially become vestigial, with the endogenous inductive fatty acid related controls however remaining. Regardless of the "true" roles of these enzymes, the fact that the enzymes themselves clearly do retain the capacity to be endogenously controlled by perturbations in fatty acid metabolism and associated hormonal changes, is very significant. This observation allows a better understanding, and predictive appreciation, of both the actions of certain xenobiotics, for example the peroxisome proliferators, as well as physiological situations in which xenobiotic metabolism, and so carcinogenesis, will change.

Chapter 4: The regulation of the mouse Cyp2e1 subfamily

Section 4.1: Introduction and Aims

In order to better understand the regulation of the CYP2E subfamily the regulation of the Cyp2e1 subfamily was studied in the mouse. The effect of chemicals previously seen to modify the levels of other P-450 was studied to ascertain any similarity in the mechanisms regulating the CYP2E subfamily and other xenobiotic metabolising P-450 families. The effect of acetone on Cyp2e1 was studied to establish the level of cross species conservation of this chemical as an inducer of the CYP2E subfamily. The levels of Cyp2e1 mRNA and protein were studied in the mouse following starvation; the changes observed were compared to the changes seen in the diabetic state in the rat. Given the suggestion that the CYP2E subfamily mRNA may become stabilised in the diabetic state (Song *et al.*, 1987), and that in other transcripts such an event has been seen to be mediated by motifs in the 3' untranslated region (UTR), the fact that the 3' UTR of the Cyp2e1 transcript has been disrupted by insertion of a β2 element is of particular significance.

As well as studies on the control of the Cyp2e1 subfamily in the whole animal the molecular basis for the regulation patterns observed was studied. The suggestion has been made that acetone stabilisation of the CYP2E subfamily protein is mediated by a substrate induced inhibition of a phosphorylation event which normally channels the CYP2E protein for rapid degradation (Ingelman-Sundberg *et al.*, 1992). This suggestion was investigated through the generation and expression of Cyp2e1 protein with mutations at the proposed controlling phosphorylation. The mutant Cyp2e1 proteins were expressed in mammalian tissue culture and the effect of these changes on the accumulation of Cyp2e1 protein in this system was studied.

The similarity between the pattern of regulation of the CYP2E subfamily and gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase, coupled with the suggestion that the CYP2E subfamily may play an endogenous gluconeogenic role (Koop & Cassaza, 1986) prompted an analysis of the possible control regions present in the 5 ' region of the Cyp2e1 gene. The 5' region of the gene was analysed for putative control elements and the possible effect of insulin on the transcriptional activity of the Cyp2e1 gene was investigated.

Section 4.2: The use of the mouse Cyp2e1 as a model system to predict the behaviour of the human CYP2E1 homologue

The products of the CYP2E1 genes from species other than mouse have been shown to be responsible for the activation of a variety of carcinogens, for example several nitrosamine (e.g. *N*-nitrosodimethylamine (NDMA) *N*-nitroso-*N*-methylmethylamide (NMMA), *N*-nitroso-*N*-butylbutylamine (NBBA), *N*-nitroso-*N*-methylbenzylamine (NMBA) and *N*-nitroso-dimethylethlyamine (NDEA) amongst others (Young & Yang, 1983), as well as a variety of hepatotoxins such as carbon tetrachloride (Johansson & Ingelman-Sundberg, 1985), benzene (Johansson & Ingelmann-Sundberg, 1988) and acetominophen (Koop *et al.*, 1982). CYP2E enzymes have also benn seen to metabolise of a variety of small solvent molecules such as alcohol (Morgan *et al.*, 1982), acetone and isopropanol (Koop & Cassaza, 1986), as well as a variety of solvent based anaesthetic molecules such as ethers, chloroform, enflurane and diethyl-ether (Pantuck *et al.*, 1988; Brady *et al.*, 1988.).

As a result of the observed metabolic roles and substrate profile of the CYP2E subfamily, it is clear that these enzymes play an important role in the potential development of chemical carcinogenesis particularly as a result of the activation of several environmentally common nitrosamines. An understanding of the regulation of the CYP2E subfamily is important to allow the prediction of situations in which changes in these metabolic activities may arise as a result the activities of enzymes in this subfamily are modified. Due to the high levels of sequence homology between the three cloned CYP2E subfamily gene sequences, both in the coding and non-coding regions, and the mouse Cyp2e1 gene, it is possible that mechanisms of control which operate on both the Cyp2e1 gene, transcript and protein can be extrapolated to the behaviour of the cognate CYP2E1 behaviour in a similar set of circumstances in other species. Thus an understanding of both chemically induced and both the physiological and pathological situations in which Cyp2e1 activities are modified in the mouse may allow an insight into situations in which similar perturbations of the human CYP2E1 activities may arise. These situations would therefore be expected to result in changes in the metabolism of a range of biologically relevant compounds which form the substrates for the CYP2E subfamily.

Initially the effect of chemicals and starvation on Cyp2e1 levels in the mouse were assessed in whole animal studies; secondly the potential molecular basis for these controls, both at the level of the Cyp2e1 protein and the gene, were studied.

Section 4.3: Chemical inducers of the CYP2E subfamily

It has long been known that ethanol produces a profound effect not only on the rate of its own metabolism, but also the levels of P-450 enzymes in the liver (Lieber & DeCarli, 1968). Ethanol exposure was noted to lead to proliferation of the smooth endoplasmic reticulum and an increase in the levels of certain metabolic activities such as aniline hydroxylation, an enzymatic activity now associated with the CYP2E subfamily (Rubin *et al.*, 1968). It is now known that small solvent molecules such as ethanol, acetone and isopropanol increase the enzymatic activity of the CYP2E subfamily and protein levels in the liver in a variety of species including the rat (Miller & Yang, 1984), chicken (Sinclair *et al.*, 1985), rabbit (Koop *et al.*, 1982), hamster Kubota *et al.*, 1986), timber wolf (Hogy & Crankshaw, 1992) and human (Perot *et al.*, 1989). The range of species in which solvents can induce members of the CYP2E subfamily suggests that this function may be universally applicable to a CYP2E homologue in any species. Solvent induction may therefore represent a common theme for the regulation of the CYP2E sufamily members metabolic activities. CYP2E protein induction by solvents was also seen to occur in the rabbit kidney (Ueng *et al.*, 1987) and bone-marrow cells (Schnier *et al.*, 1989). As discussed in connection with the diabetic induction of the CYP2E subfamily it is possible that acetone represents an endogenous substrate and inducing agent for this subfamily and that other small solvent molecules may possess their inducing capabilities by mimicking acetone both as a substrate and an inducer (Koop & Cassaza, 1986, Section 3.10.1).

In vitro studies on the induction of CYP2E1 protein in the rat suggested that acetone, and other inducing chemicals, operated through the stabilisation of pre-existing CYP2E1 protein. As a result of gene extinction, primary rat hepatocyte cell cultures are seen to lose detectable levels of CYP2E1 mRNA and protein around three days after the culture has been established. If however CYP2E1 substrates are added to the culture medium, although the CYP2E1 gene still becomes extinct and CYP2E1 mRNA is no longer detectable, the CYP2E1 protein levels are maintained at 75% of the original level, whilst control cells grown in the absence of CYP2E1 substrates lose 85% of the original CYP2E1 protein level. A correlation was seen between the level of activity of CYP2E1 to the various substrates and the level of CYP2E1 protein retention which they generated; the more tightly a substrate bound the CYP2E1 protein, as measured spectrophotometrically, the better its ability to maintain the CYP2E1 protein levels in the primary culture system. These results suggest that the substrates generate CYP2E1 induction by binding to the CYP2E1 protein and potentially render it resistant to a normal degradative process (Eliasson *et al.*, 1988).

These *in vitro* results matched those obtained in *in vivo* studies. In the rat it was seen that the induction of the CYP2E1 protein and its associated metabolic activities was associated with an elevation in CYP2E1 protein levels without an increase in CYP2E1 mRNA level (Song *et al.*, 1986). The increase in the level of the CYP2E1 protein and associated biochemical activities in the rat liver were seen to be quickly reversed following removal of the inducing chemical, for example the ethanol-induced increase in *p*-nitrophenol hydroxylase activity, an activity associated with the CYP2E subfamily, was seen to be lost one day after ethanol withdrawal (Hetu & Joly, 1985). These studies suggest that the CYP2E1 protein is stabilised in the whole animal in a similar manner to that observed in tissue culture systems and that the protein is rapidly turned over in the hepatocyte.

By labelling newly synthesised proteins with a pulse of radioactive amino-acid precursors and then immuno-precipitating the CYP2E1 protein, it was possible to observe the effect produced by solvent molecules on the turn over of the CYP2E1 protein in the rat liver. It was seen that in control animals CYP2E1 protein was degraded in a biphasic manner consisting of a rapid and slower turnover pathway, generating a CYP2E1 protein half-life of around 7 hours and 37 hours respectively. In the acetone-treated animals it was seen that the rapid CYP2E1 turnover pathway was exhibiting a reduced level of degradative activity and that the majority of the CYP2E1 protein was lost via the slower degradative system. As a result of this change in degradation pathways a longer half-life for the CYP2E1 protein was observed (Song *et al.*, 1989).

It is possible thatthe induction of CYP2E1 protein could result from either stabilisation of the pre-existing protein or increased translation of the message. By monitoring the incorporation of a radioactive pulse into CYP2E1 protein in control and acetone treated animals it was seen that the specific radioactivity was higher per unit of CYP2E1 protein in the control animals than in the treated animals. This suggests that the chemical induction was not due to an increase in translation as in the acetone-induced animals the non-radioactive labelled pre-existing CYP2E1 proteins which become chemically stabilised effectively dilute the newly synthesised CYP2E1 proteins (Song *et al.*, 1989).

These studies suggest that the CYP2E subfamily of many species contains a common regulatory theme of induction by substrate molecules. This induction appears to be the result of substrate-induced protein stabilisation and may operate through the blocking of a rapid degradative system acting upon the CYP2E subfamily proteins in the control state. This mechanism of control may also extend to physiological and pathological situations in which the levels of CYP2E subfamily member activities are seen to be regulated.

Section 4.4: Induction of Cyp2e1 by chemicals in the mouse

Section 4.4.1: The effect of chemicals known to induce other P-450 families on the level of Cyp2e1 protein in the liver

The effect of chemicals seen previously to produce dramatic effects on the protein levels of other P-450 families and other proteins associated with drug metabolism on the level of Cyp2e1 protein in the mouse liver was investigated. Mice were treated with β-naphthoflavone, 3-methylcholanthrene (CYP1 family inducers), dexamethasone, phenobarbital and 1, 4-bis[2-(3,5-dichloropyridyloxy) benzene] (TCPOBOP), 2-acetylaminofluorene, pyrazole and aflatoxin B1 (CYP2 and 3 family inducers), and *N*-nitrosodimethylamine (NDMA), as detailed in Section 2.16.2a. Protein was prepared from the livers and the effects of these inducers on Cyp2e1 protein levels was assessed using a polyclonal antibody raised to purified rat CYP2E1 protein; polyclonal antibodies raised to rat CYP2B1 were also used to demonstrate the effects of some of the chemicals on another CYP2 subfamily.

It was seen that no chemical treatment produced any profound effect on the level of Cyp2e1 protein (Figure 4.1); the nature of the lower molecular weight band seen below the Cyp2e1 protein is discussed below. By comparison the levels of protein in the Cyp2b subfamily can be seen to have become elevated by phenobarbital, dexamethasone, 3-methylcholanthrene, phenobarbital, 1 , 4-bis [2 - (3, 5 - dichloro-pyridyloxy) benzene] (TCPOBOP), and 2-acetylaminofluorene. NDMA, a substrate of the CYP2E subfamily, generated a drop in the level of Cyp2e1 protein but also caused a marked degeneration of the liver; in this respect it is interesting however that the levels of Cyp2b can be seen to be elevated. In conclusion Cyp2e1 levels appear to be unaffected by chemicals which markedly induce the levels of a variety of other P-450 subfamily members (Figure 4.1).

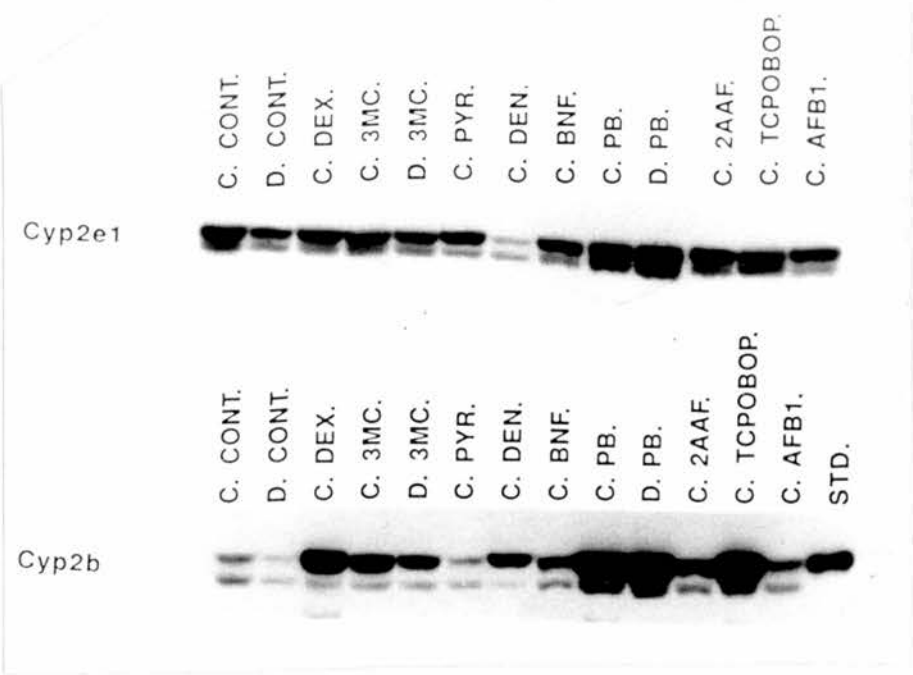


Figure 4.1: The effect of known inducers of P-450 protein on the level of Cyp2e1 protein in the mouse liver. Hepatic microsomal protein was isolated from C57BL/6 (**C**) and DBA/2N (**D**) male mice treated with the following compounds: **DEX.**: Dexamethasone, **3MC:** 3-methylcholanthrene, **PYR:** Pyrazole, **DEN:** *N*-nitrosodiethylnitrosamine, **BNF:** β-napthoflavone, **PB:** Phenobarbital, **2AAF:** 2-acetylaminofluorene, **TCPOBOP:** 1, 4- bis [2 - (3, 5 -dichloropyridyloxy) benzene], **AFB1:** Aflatoxin B1. Actual tretment regimes are detailed (Section 2.16.2).15μg of microsomal protein was separated by SDS/PAGE on 12% gels, transferred to nitrocellulose filters and probed with polyclonal antibodies raised to purified rat CYP2B1 and CYP2E1 protein; the CYP2B1 standard (**STD.**) was purified from rat liver. The P-450 proteins migrated with a M_r of approximately 52,000 and no bands were detected other than those shown.

Section 4.4.2: Induction of Cyp2e1 by acetone

It would be predicted that, in view of the very high levels of amino-acid sequence conservation between the mouse Cyp2e1 and CYP2E1 proteins of other species that acetone would lead to an induction of the level of Cyp2e1 protein in the mouse. To test this postulate male and female DBA/2N mice were fed 1% (v/v) acetone in their drinking water for 14 days. Protein was prepared from a variety of organs from both control and acetone treated animals and the effect of the chemical treatment investigated.Acetone treatment could be seen to generate an increase in the level of Cyp2e1 protein in the liver, kidney, lung, spleen, oesophagus, testes and pancreas; essentially anywhere that the Cyp2e1 protein could be immunologically detected it was seen to become elevated (Figure 4.3). Cyp2e1 could not be detected using this technique in the brain, heart, intestine, stomach or striated muscle. From these results it is apparent that acetone potentially can penetrate throughout the body and lead to an increase in the level of Cyp2e1 protein in any tissue which expresses it (Figure 4.3).
The observation that the CYP2E subfamily is present, and acetone-induced, in the oesophagus is of particular note given the established link between alcohol consumption and oesophageal cancers in humans (Lieber & Garro, 1990). The incidence of oesophageal cancer is particularly high in certain geographical regions noted for the local production of drinks with a high nitrosamine content, an example of this being Calvados, the apple-based brandy from the Calvados Department of Normandy (Tuyns *et al.*, 1987). Immunohistochemical studies of the female oesophagus, in which the cross-reacting band is absent, clearly demonstrate the extent of the Cyp2e1 protein induction by acetone in this tissue (Figure 4.4).
These studies illustrate a sexual dimorphism in the level of expression of Cyp2e1 protein in both the lungs and kidneys, with much lower levels being seen in the male and female organs respectively (Figure 4.2); the significance and possible reasons for this observation are discussed below.
The polyclonal antibody employed in these studies also recognises a slightly lower molecular weight protein running just below the Cyp2e1 protein on the acrylamide gels in preparations from some tissues. The cross-reacting band is however not present in other tissues for example the kidney, pancreas and spleen, and is clearly sexually dimorphic, if not dependent, in the oesophagus (Figure 4.3). It is likely that this band represents a P-450 protein distinct from Cyp2e1 but sharing a common epitope(s). The cross-reacting band has been noted in other studies employing this rat polyclonal antibody on certain mouse protein preparations (Hong *et al.*, 1989); in this study it was suggested to represent a degradation product of the Cyp2e1 protein (Hong *et al.*, 1989). From the pattern produced by this second band in the study of the effect of a

range of chemical inducers it can be seen that the lower band demonstrates some of the inductive behaviour of the CYP2B subfamily to certain compounds (Figure 4.1). The lower band is decreased by acetone treatment in the liver and lung (Figure 4.3); in a study on the effect of ethanol treatment on the levels of a variety of P-450 in the rat liver it was seen that an increase in the level of benzephetamine *N*-demethylase, a biochemical activity associated with the CYP2B subfamily, was observed after withdrawal of ethanol suggesting that a members of this subfamily may be repressed by acetone treatment (Hetu & Joly, 1985). If this band represented a degradation product of the Cyp2e1 protein it may be expected to be seen in all tissues expressing Cyp2e1; collectively these observations suggest that the lower cross-reacting band represents a P-450 protein distinct from Cyp2e1, potentially a member of the Cyp2b subfamily, which shares a common epitope(s) with Cyp2e1.

Acetone treatment did not lead to a concomitant increase in the level of Cyp2e1 mRNA, suggesting that the Cyp2e1 protein induction does not result from increased transcriptional activity of the Cyp2e1 gene. The level of Cyp2e1 mRNA was seen to decrease in the female kidney upon acetone treatment (Figure 4.5). Decreased CYP2E mRNA levels have been observed in the rabbit (Porter *et al.*, 1989), and rat hetatic tissues following chemical induction, and a similar observation was made in the BB/E diabetic male rat kidney (Section 3.4); in the rat liver the message level was seen to drop by 50% following chemical induction (Kim & Novak, 1990. Kim *et al.*, 1990). Reductions in the level of the CYP2E subfamily mRNA may be as a result of a potential increase in the translation initiation from the message leading increased mRNA (Section 3.7.5).

The Cyp2e1 protein behaves as would be predicted on treatment with acetone and consistent with the idea that the presence of a substrate stabilises pre-existing Cyp2e1 protein. No increase in the level of Cyp2e1 mRNA is seen on chemical induction with acetone. A fall in the level of Cyp2e1 mRNA is seen in the female kidney suggesting that a modification in the translatability of the Cyp2e1 mRNA may be occurring.

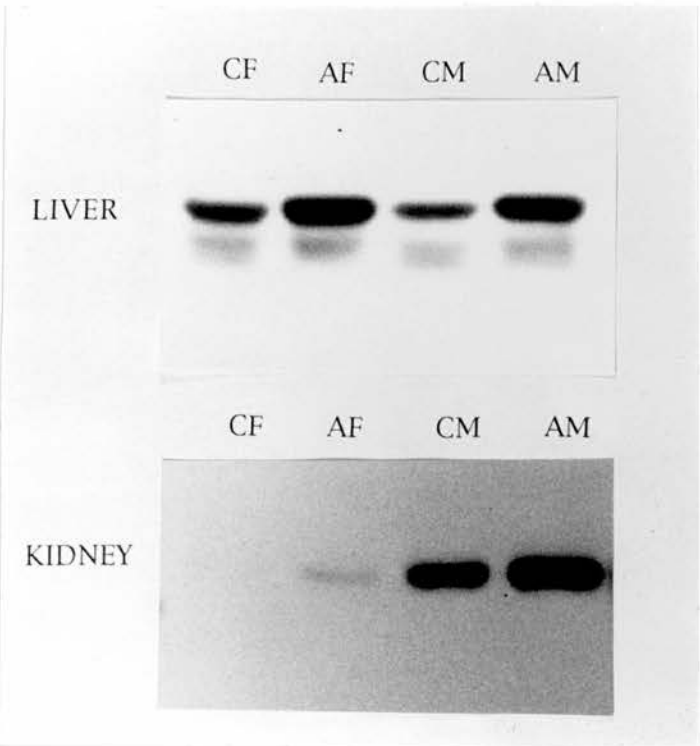


Figure 4.2: The effect of acetone treatment on the level of Cyp2e1 protein in the mouse liver and kidney. Microsomal protein was isolated from control (**C**) and acetone-treated (**A**), male (**M**) and female (**F**) DBA2/N mice tissues as indicated. 15μg of protein were separated by SDS/PAGE on 12% gels, transferred to a nitrocellulose filter and probed polyclonal antibodies raised to purified rat CYP2E1 protein. No bands were detected other than those shown and the Cyp2e1 protein migrated with a M_r of approximately 52,000.

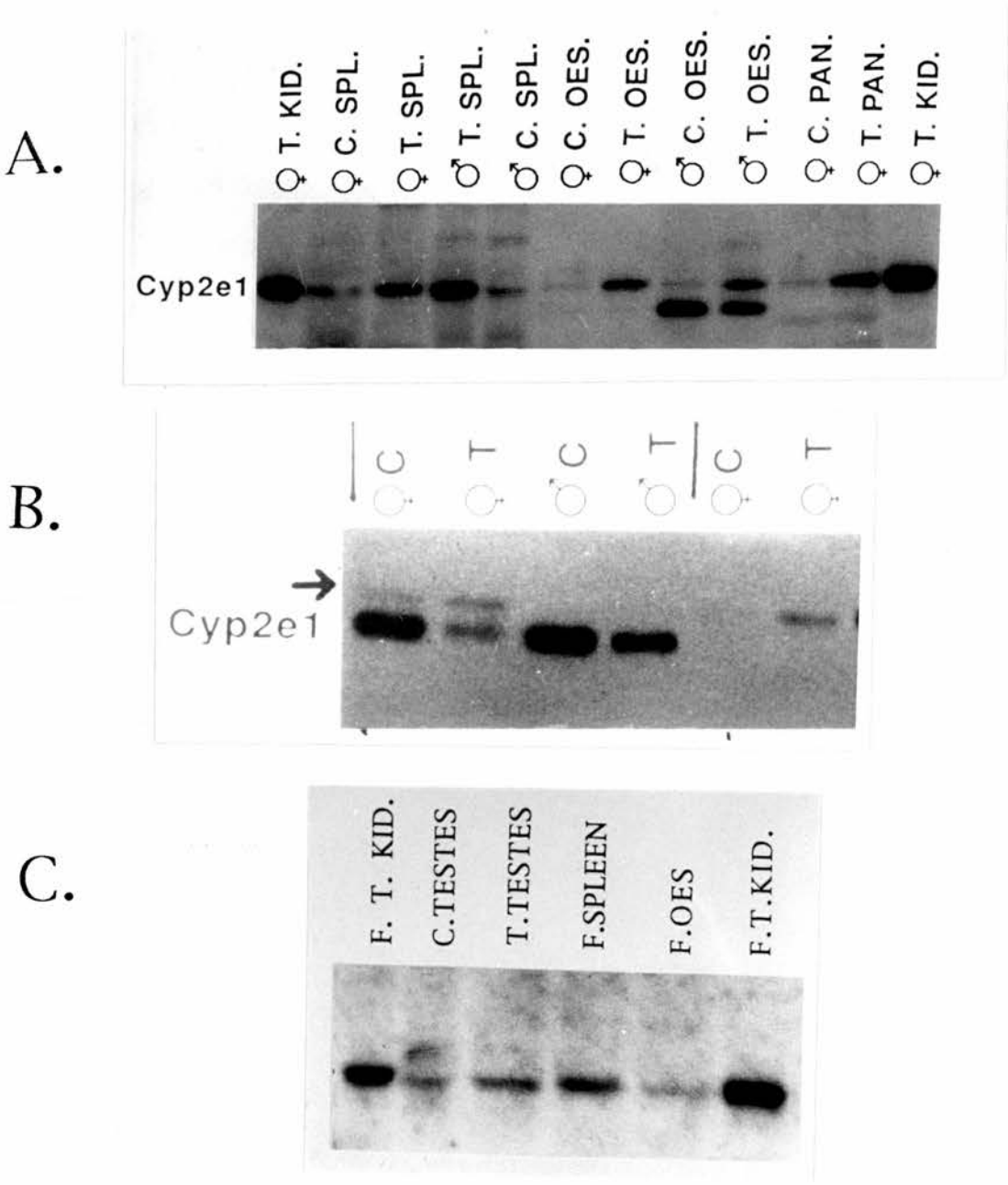


Figure 4.3: The effect of acetone treatment on the level of Cyp2e1 protein in a variety of mouse tissues. **A:** Microsomal protein was isolated from control (**C**) and acetone-treated (**T**), male and female (as indicated) DBA2/N mouse kidney (**KID.**), spleen (**SPL.**), oesophagus (**OES.**), and pancreas (**PAN.**). 15μg of kidney and 100μg of other tissue proteins were loaded per track. **B:** Microsomal protein was isolated from control (**C**) and acetone-treated (**T**), male and female (as indicated) DBA2/N mouse lung (left hand 4 tracks) and female kidney (right hand 2 tracks). 15μg of each sample was loaded per track. The arrow indicates the Cyp2e1 protein and the lower cross-reacting band is discussed in the text. **C:** Microsomal protein was isolated from control (**C.**) and acetone-treated (**T.**) DBA2/N mouse testes, female (**F.**) spleen and oesophagus (**OES.**) and 100μg was loaded per track together with 10μg of female acetone-treated kidney (**F. T. KID.**). In each instance the samples were separated by SDS/PAGE on 12% gels and the protein transferred to nitrocellulose filters. The filters were probed using polyclonal antibodies raised to purified rat CYP2E1 protein. The Cyp2e1 protein migrated with a Mr of approximately 52,000 and no bands were detected other than those shown.

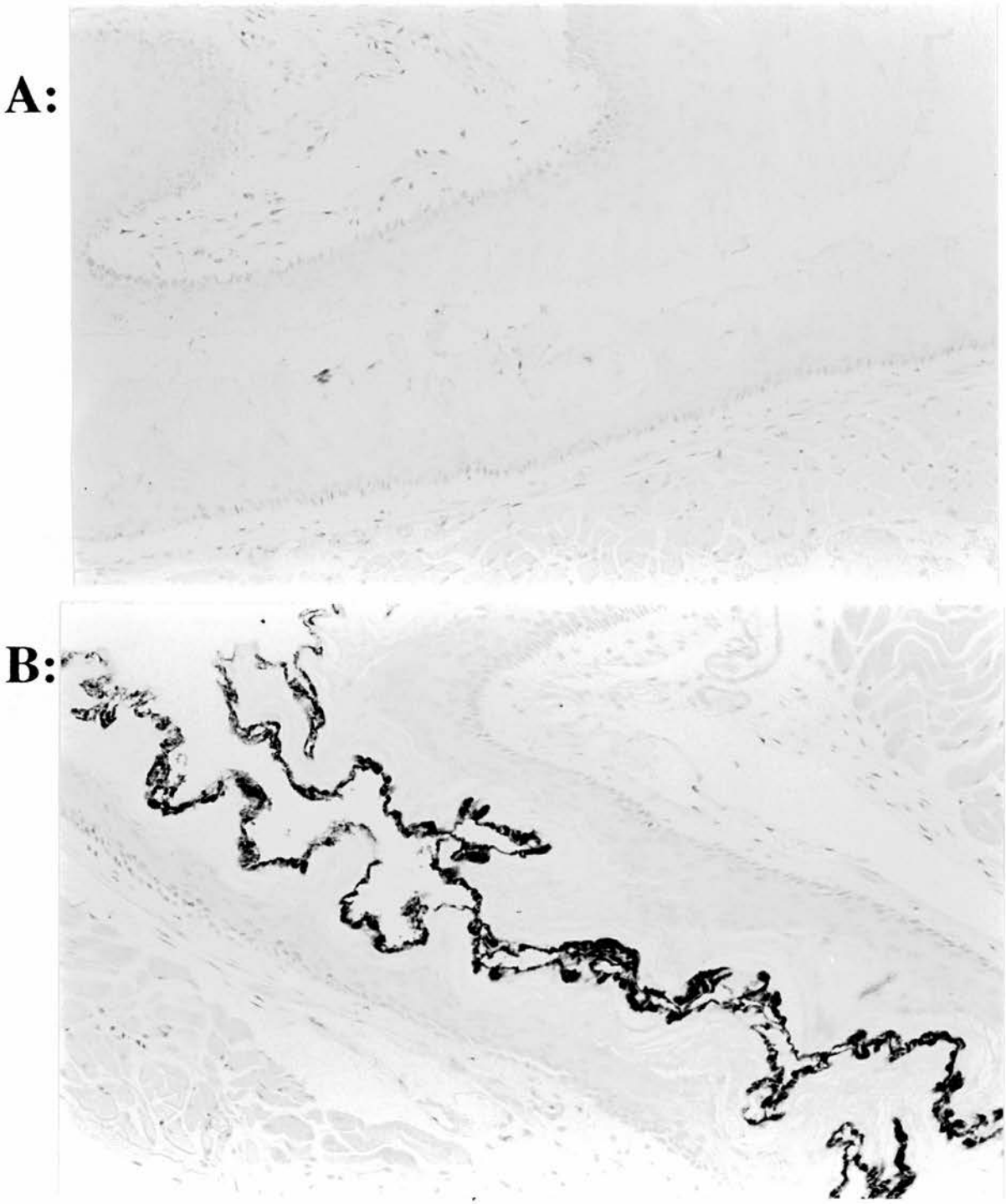


Figure 4.4: Immunohistochemical localisation of the Cyp2e1 in the female DBA2/N mouse oesophagus. The presence, and acetone-induction, of the Cyp2e1 protein in the oesophagus was determined using polyclonal antisera raised to purified rat CYP2E1 protein. The antibodies recognising the Cyp2e1 protein were complexed with biotinylated swine anti-rabbit antisera and the immune-complex detected using an avadin-biotin peroxidase detection system; the reaction of this system with its substrate, 3, 3-diaminobenzidine, gives the dark staining seen indicating the localisation of the Cyp2e1 protein. **A:** Control animal. **B:** Acetone treated animal. This analysis was performed in collaboration with Dr D. J. Harrison and Mrs H. Wolf, Department of Pathology, Edinburgh University.

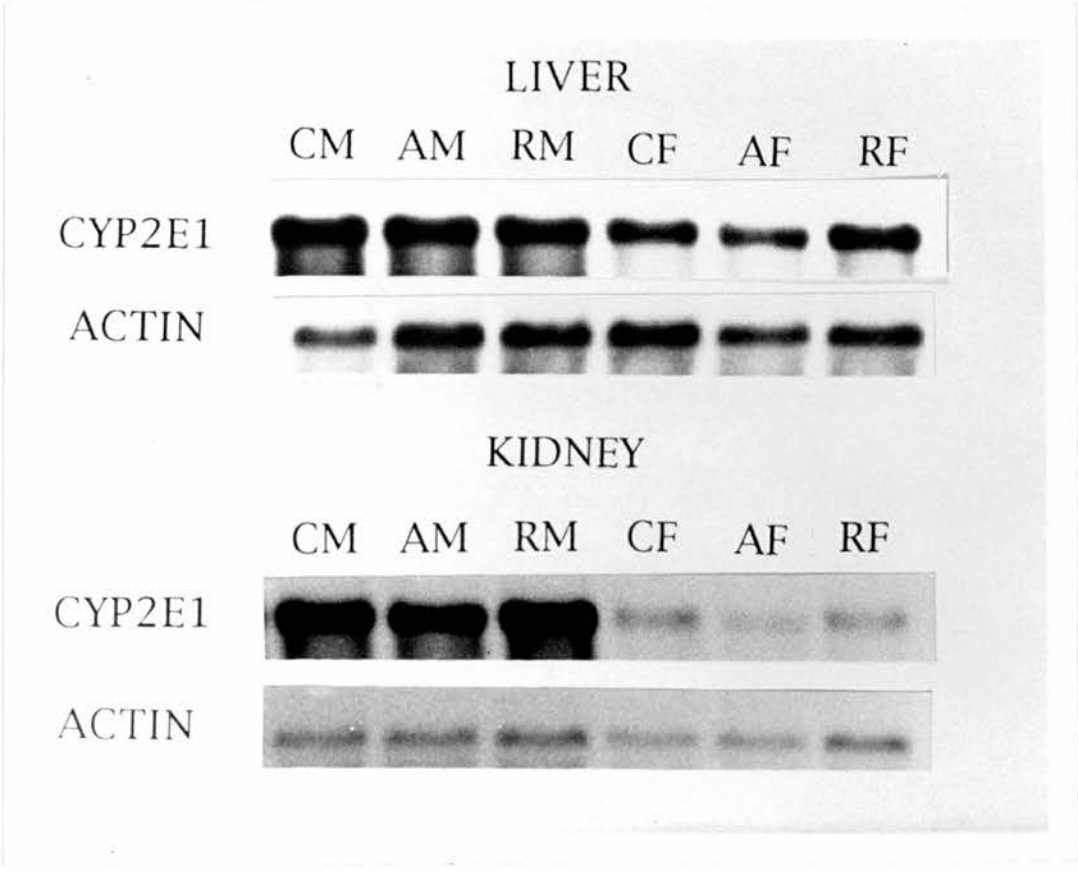


Figure 4.5: The effect of acetone treatment on the levels of Cyp2e1 mRNA in the liver and kidney. Total RNA was isolated, using the tissue indicated, from control (**C**) male (**M**) and female (**F**) DBA2/N mice, animals treated with acetone (**A**), and animals treated with acetone and then returned to normal conditions (**R**) (Section 2.16.2a). 10μg of total RNA was separated on an agarose gel and transferred to a nylon membrane by capillary action. The filter was hybridised with radioactive probes generated from a mouse partial Cyp2e1 and β-actin cDNA respectively. The Cyp2e1 transcript and β-actin transcript migrated with a size of approximately 1,800 and 1,000 bases respectively and no bands other than those shown were present.

Section 4.5: Induction of Cyp2e1 by starvation

The possible endogenous substrate and inducing agent of the CYP2E subfamily enzymes may be acetone. Acetone is produced as a result of elevated fatty-acid β-oxidation in response to modifications in the ambient fuel sources available. Such elevations occur in diabetes and starvation; in this respect diabetes could be looked upon as being an extreme form of starvation. The CYP2E subfamily is suggested to have an endogenous gluconeogenic role in these situations allowing the conversion of acetone indirectly to glucose (Koop & Cassaza, 1986), and the observed pattern of its induction in these and the chemically induced situations can be rationalised in connection with this role.

In both the diabetic state and as a result of starvation, an elevation of both CYP2E1 mRNA and protein has been observed in the rat (Young & Yang, 1982; Miller & Yang, 1984; Section 3.4.1). These observations are reflected biochemically by the increased activation of carcinogens known to be metabolised by CYP2E1 in the starved animal, with levels of NDMA demethylation, for example, being enhanced by between 50 and 100% following starvation in rats (Venkatensan *et al.*, 1970).

Section 4.5.1: The mechanism for induction of CYP2E1 in the starved and diabetic states; the role of acetone and the determination of message stability

The induction of the CYP2E subfamily in the starved and diabetic states may relate in part to the elevation of ketone bodies leading to the stabilisation of the enzyme by its substrate. This observation cannot account for the elevation in CYP2E1 mRNA as such an event was not seen in chemical induction alone. In the rat it was calculated that the acetone level increase alone could not proportionately account for the levels of CYP2E1 protein increase seen, and that the induced message levels were not therefore a passive feature of the starved state (Miller & Yang, 1984). The increase in CYP2E1 mRNA has previously been suggested to be the result of stabilisation of existing messages (Song *et al.*, 1987); however, possible problems concerning this suggestion have bee discussed (Section 3.7.3).

To date most mRNAs which have been seen to be controlled by regulatable stability have been shown to possess structural features within the untranslated regions (UTRs) of the message, principally the 3' UTRs as these portions of the message are free from the restraints present in the coding portions of the message. The 3' UTR can therefore potentially generate structural motifs and contain recognition sequences bound by regulatable protein components, leading in turn to differential degradation of the message. Examples of this mechanism of control are seen in the transferrin-receptor

(Owen *et al.*, 1987), phosphoenolpyruvate carboxykinase (Petersen *et al.*, 1989) and certain histone (Levin *et al.*, 1987) mRNAs.

In general terms, as well as in the specific cases of mRNA possessing regulated degradation, the stability of a message has been seen to be determined by the nature of its 3'UTR. Messages can have a half-life of several days, as seen for example in the globin genes, to only a few minutes, as seen in the case of many messages involved in the control of cellular division. UAAAU motif sequence elements in rapidly degraded messages, for example *c-fos*, dictate this rapid turnover; in this instance components present in *c-fos* which lead to its rapid turnover are seen to be absent in the oncogenic equivalent *v-fos* and the resulting mRNA is therefore much more stable. However, by fusing the 3' UTR of *c-fos* onto *v-fos* the rapid degradation of the message returns (Miller *et al.*, 1984). Similar transfers of message stability on transfer of heterologous 3' UTR sequences have been seen in several experiments. For example the exchange of a human short half-life lymphokine mRNA's 3' UTR with the long half-life globin mRNA's 3' UTR leads also to a reciprocal exchange of message stabilities (Shaw & Kamen, 1986). The main conclusion of these and other related experiments is that one of the major determinants for the half-life of a mRNA resides in the 3' UTR and its associated poly-(A) tail (See also Section 3.7.4).

It would be expected that Cyp2e1 protein would behave in a similar manner to that observed in other species in starvation if the suggestion that acetone plays a role in its induction in this situation is correct. What is not clear however is how the Cyp2e1 mRNA would behave in starvation given the suggestion that the induction observed in the diabetic rat is the result of CYP2E1 mRNA stabilisation. The endogenous Cyp2e1 3' UTR has been ablated following the insertion of a β2 short interspersed repetitive element (SINE, Section 5.4.1). If the control of the CYP2E subfamily message stability is executed by previously recognised mechanisms involving the 3' UTR, this control may be lost in the mouse Cyp2e1 mRNA. In this respect the mouse offers an excellent *in vivo* genetic model to investigate the potential nature of the CYP2E subfamily message regulation. If the β2 SINE insertion event had not happened naturally to generate a model system of this kind producing a Cyp2e1 message without a 3' UTR by conventional transgenic techniques would take a long time both to engineer and assay. The effect of starvation in the mouse on the Cyp2e1 protein and mRNA levels was investigated to address these possibilities.

Section 4.5.2: The effect of starvation on the Cyp2e1 protein and mRNA

The effect of starvation on the hepatic level of Cyp2e1 mRNA and protein was studied in male and female mice over a period of starvation extending for both 12 and 24 hours. Protein was isolated from the control and starved animal groups and the level of Cyp2e1 protein assessed by Western blott analysis; the level of Cyp2e1 protein is seen to become elevated upon starvation (Figure 4.6).

Total RNA was isolated from control and the two starved animal groups, was separated on denaturing agarose gels and transferred to nylon membranes; the resultant memebrane was hybridised with the 800 base-pair partial mouse Cyp2e1 cDNA probe (Section 5.2). Starvation leads to an elevation in the level of hepatic Cyp2e1 mRNA in both male and female mice; it is also apparent that longer term starvation (24 hour) leads to a higher elevation than that seen by shorter term (12 hour) starvation. The cDNA for the rat microsomal glutathione S-transferase (GST) was used as a control probe to demonstrate equal loading of the total RNA; as discussed (Section 3.6) the microsomal GST potentially represents a good control probe for this purpose as it was seen to be unaffected by the diabetic state in the rat and so may be expected to be unaffected by starvation in the mouse (Figure 4.6).

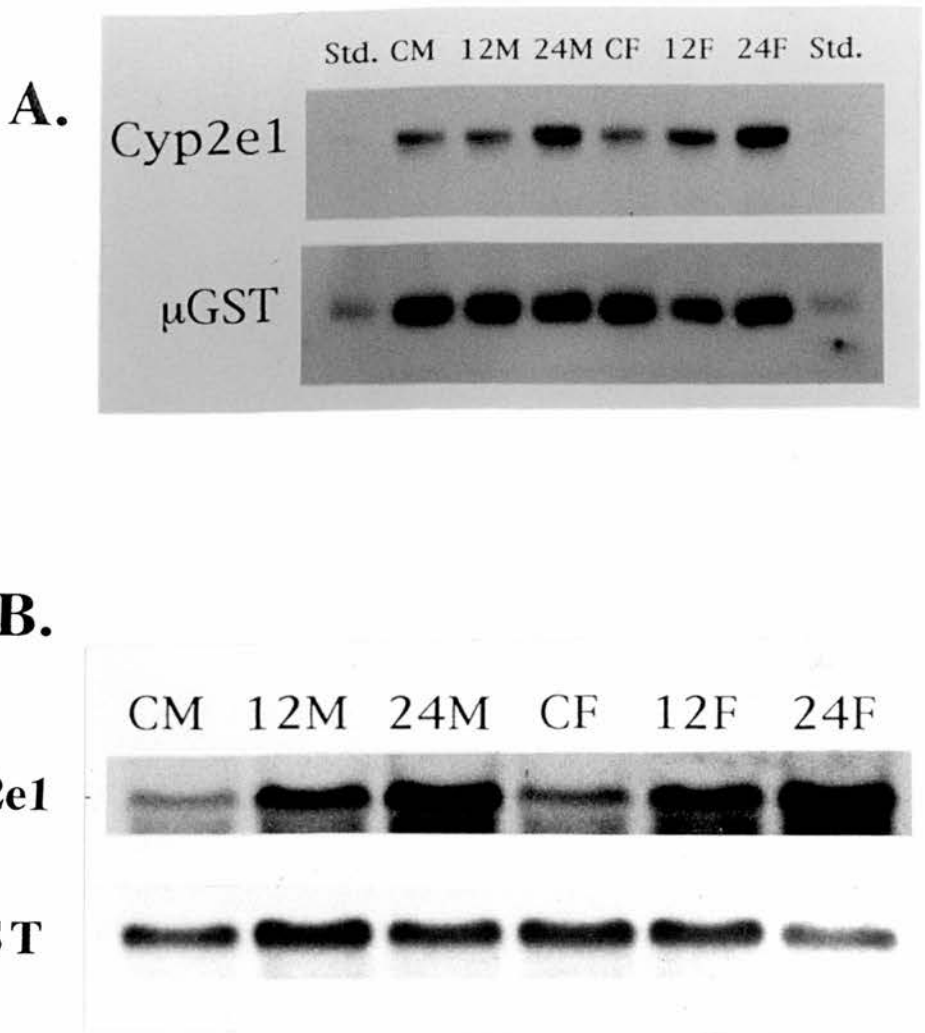


Figure 4.6: The effect of starvation on the level of Cyp2e1 mRNA and protein in the mouse. **A:** 10 μg of hepatic microsomal protein isolated from male (**M**) and female (**F**) control (**C**), 12 hour starved (**12**) and 24 hour starved (**24**) DBA2/N mice were saparated by SDS/PAGE on a 12% and 9% gel. The proteins were transferred to nitrocellulose filters and the proteins separated on the 12% and 9% gels were probed with polyclonal antisera raised to purified rat CYP2E1 and microsomal GST (μGST) proteins respectively. The μGST standard (**Std.**) was prepared from purified μGST protein hetrologously expressed in *E. coli*. The Cyp2e1 protein and μGST protein migrated with a M_r of approximately 53,000 and 17,000 respectively and no bands other than those shown were detected. **B:** Total RNA was isolated from the livers of DBA2/N mice, identified as detailed above, separated on an agarose gel and transferred to a nylon membrane by capillary action. The filter was hybridised with radioactive probes generated from a mouse partial Cyp2e1 and a rat cDNA respectively. The Cyp2e1 transcript and μGST transcript migrated with a size of approximately 1,800 and 950 bases respectively.

Section 4.5.3: The implication of the elevation of the Cyp2e1 mRNA in the starved state

The Cyp2e1 mRNA was seen to be elevated in starvation; previous observations of CYP2E1 mRNA elevations in chemically-induced diabetic rats have been attributed to a stabilisation effect of the diabetic state on pre-existing CYP2E messages and so it was suggested that similar mechanism operated in starvation (Song *et al.*, 1987). Possible problems relating to this conclusion have been discussed (Section 3.7.3), and the observation that the mouse Cyp2e1 mRNA levels are elevated in starvation adds further doubt to a role for a stabilisation event in the control of CYP2E mesage levels in general.

The Cyp2e1 does not possess its endogenous 3' UTR, but an inserted β2 retrotransposon and its accompanying poly-(A) addition signal; if a regulatable stability of the CYP2E subfamily messages is to be executed by conventional mechanisms then a role for the 3' UTR in this process would be expected and the observed Cyp2e1 mRNA elevation in starvation would not be predicted. Unless a stabilisational event is mediated as a result of elements contained within the residual endogenous Cyp2e1 3' UTR or other areas of the CYP2E subfamily transcripts, or via a previously unknown stabilisation mechanism, this observation provides a strong genetic argument for the induction of the CYP2E messages to occur via other means.

The fact that prolonged starvation produced a further elevation of both the Cyp2e1 mRNA and protein levels may reflect the severity of the starved state at the two time points; a larger proportion of cells in the liver may be affected by starvation in the 24 hour than in the 12 hour starved samples resulting in a proportionate increase in the Cyp2e1 mRNA and protein levels in each instance.

A report on the effect of starvation on the induction of NDMA-demethylase activity associated with CYP2E1 subfamily, in the rat liver showed that no elevation was seen in the presence of the RNA synthesis inhibitor actinomycin D. This observation would suggest a possible role for transcriptional activation of the CYP2E1 gene in the generation of CYP2E1 mRNA elevation as the result of starvation (Young and Yang, 1982). Transcriptional activation of the CYP2E subfamily would agree with one interpretation of the results obtained in connection with the elevated CYP2E1 mRNA levels in the diabetic state in the rat (Section 3.7.8).

Section 4.6: Other controls of the Cyp2e1 subfamily: Sex hormones and the Pituitary

Cyp2e1 is clearly sexually dimorphic in the mouse kidney, with higher levels being expressed in the male than in the female (Figure 4.3). The sexual dimorphism in Cyp2e1 levels may be reflected in the observed sexually dimorphic effects of several carcinogenic nitrosamines on the mouse kidney with neoplasms predominating in the male kidney in every instance (Noronha, 1977. Hong *et al.*, 1980; Mohla, 1981; Hawke & Welch, 1985; Ampy & Williams, 1988; Hong *et al.*, 1989). It was noted that chloroform is relatively much more toxic to the male than the female kidney in the mouse (Pohl *et al.*, 1984) which also could potentially relate to the sexual dimorphism in Cyp2e1 levels in this tissue.

Sexually dimorphic and sexually dependent P-450 levels are observed in the rat and these differences are thought to be attributable to differences in the patterns of growth hormone secretion between the two sexes (Section 3.7.8). In mice a sexually dimorphic pattern of growth hormone is seen with male animals having, in a manner similar to the rat, a more stable continuous level of growth hormone (MacLoed *et al.*, 1991). It is possible to modify the levels of growth hormone present in mice both by the use of hypophysectomised animals and genetic model strains (MacLeod & Shapiro, 1989. Norstedt & Palmiter, 1984). In this laboratory the "little" mouse strain, a C57BL/6 derived strain deficient in the pituitary receptor for growth hormone releasing factor and consequently displaying a 90 to 95% decrease in growth hormone levels, the effect of the fall in growth hormone on the renal Cyp2e1 protein level was analysed. As well as sexual dimorphism occurring in Cyp2e1 protein levels, it was also noted for members of the Cyp4a, Cyp2b, Cyp3a, and Cyp2c subfamilies. The little mice studies however, suggested that growth hormone played no part in the generation of the Cyp2e1 sexual dimorphism, or that of any other P-450 subfamily, as the levels of this and the other P-450 proteins were unchanged in the growth hormone deficient little-mouse strain relative to its control (Henderson *et al.*, 1990). Growth hormone has been suggested to regulate the levels of Cyp2a4 protein in a separate study however (Noshiro & Negishi, 1986).

The "dwarf" rat has similarly low levels of circulating growth hormone and so can be compared to the little-mouse strain as a model for the effect of growth hormone. Using the dwarf-rat strain no changes in the sexually dependent P-450 patterns in the CYP2C subfamily are observed, leading to a similar suggestion that growth hormone played no role in the establishment of sex differences in this subfamily in the rat liver (Bullock *et al.*, 1991). In a different interpretation of the same dwarf rat model it was seen that, although the absolute levels of growth hormone were reduced, the sexually dimorphic secretory pattern of the enzyme was retained and so the CYP2C enzyme profiles were

unaffected (Shapiro *et al.*, 1989). In a similar manner the secretory pattern of growth hormone in the little mouse strain may be maintained and so a role for growth hormone can not be ruled out based on this model system alone.

In a separate study employing hypophysectomised mice it was seen that the complete removal of growth hormone by this technique had no effect on the regulation of the Cyp2e1 protein in the mouse liver although it did not address the dimorphism in the kidney (Hong *et al.*, 1990). This observation is significant as a similar treatment in the rat leads to an elevation of both the CYP2E1 mRNA and protein levels (Yamazoe *et al.*, 1989a). Hypophysectomy also effects the levels of several other P-450 in the rat; levels of CYP1A, CYP2B as well as CYP2E were seen to be elevated in the hypophysectomised rat (Williams and Simonet, 1988. Yamazoe *et al.*, 1989a). As discussed (Section 3.7.8) the levels of circulating growth hormone, both in terms of peak height and amplitude, are decreased in starvation in the rat and similarly in diabetes (Tannenbaum, 1979. Tannenbaum *et al.*, 1981.). It was suggested that the fall in growth hormone levels in diabetes and starvation may be responsible for the elevated CYP2E1 mRNA levels seen in these situations (Yamazoe *et al.*, 1989a). If growth hormone does not generate the same effect on levels of Cyp2e1 mRNA or protein in the mouse as it is seen to do in the rat, either a different control mechanism is operating in this species or the changes in growth hormone level in the rat are coincidental to other common, and unknown, modifications occuring in starvation in both species. In humans, growth hormone levels are elevated by both starvation (Hansen *et al.*, 1970; Ho *et al.*, 1988) and diabetes (Asplin *et al.*, 1989) but CYP2E1 levels were seen to be elevated (Song *et al.*, 1990) which would not be expected if an analogy to the apparent effect of growth hormone on rat CYP2E1 levels applied. Alternatively, differences exist in the promoter regions between the rat, human and mouse genes and these may account for differences in transcriptional control mechanisms between species (Section 4.12); if this is the case it may be that the control of Cyp2e1 in the mouse may more closely resemble that of the human CYP2E1.

Clear results were obtained regarding the role of the sex hormones in the sexual dimorphism of Cyp2e1 in the mouse kidney. Castration of male mice led to the generation of a female renal Cyp2e1 level and this effect was reversed by administration of testosterone; similarly the female renal Cyp2e1 protein levels were seen to be elevated by testosterone administration (Henderson *et al.*, 1990). Using the "testicularly-feminised" (Tfm) mouse strain, which have defective androgen receptors, it was seen that a female-like renal Cyp2e1 protein level was present in the males. This lower Cyp2e1 protein level could not be reversed directly by testosterone suggesting that the androgen receptor pathway plays a role in the establishment of the Cyp2e1 dimorphism (Henderson & Wolf, 1991). Elevations in renal Cyp2e1 levels in female

mice took around eight days to occur following testosterone administration, suggesting that testosterone mediates its effect indirectly through changes in the expression of other genes rather than exerting a direct effect on the Cyp2e1 gene itself (Henderson & Wolf, 1991). The testosterone-mediated induction of Cyp2e1 in the female kidney was shown to be transcriptional, however, testosterone produced no changes on the levels of Cyp2e1 protein or mRNA in the female liver (Hong *et al.*, 1989).

The generation of the observed Cyp2e1 sexual dimorphism demonstrates that elements controlling the transcription of the Cyp2e1 gene can be regulated. The observed lack of effect of growth hormone on Cyp2e1 transcription in the mouse, compared to its seemingly repressive action on the CYP2E1 gene in the rat, either highlights species specific differences in the mechanisms of the control of the genes or that growth hormone level changes in the rat are coincidental to concommitant changes in another effector in both species.

Section 4.7: The molecular nature of the CYP2E subfamily substrate induced protein stabilisation

Substrate induced protein stabilisation appears to be a common theme in the regulation of CYP2E subfamily members. In chemical induction, as well as in starvation and diabetes where the CYP2E subfamily proteins are elevated, a role for substrate induced stabilisation has been suggested. Behind this observation is the suggestion that the rationale for the CYP2E subfamily induction pattern relates to the endogenous role of the protein in a gluconeogenic pathway. All situations where the CYP2E subfamily members become elevated can thus be seen to relate to either an elevation in the level of acetone, the proposed endogenous substrate, or the mimicking of acetone's actions by other chemicals. It is suggested by both *in vivo* (Song *et al.*, 1989) and *in vitro* approaches (Elliasson *et al.*, 1988) that the presence of the endogenous substrate, or a mimic, reduces the effect of a fast acting component of the degradative pathway normally responsible in part for the CYP2E subfamily protein turnover.

As a result of protein turnover, degradation cannot be regarded as less significant than synthesis in the control of the steady state and inducible levels of a protein. This aspect of control has attracted less investigation however, due to the technical difficulties associated with it by comparison to the more readily studied *de novo* synthesis of inducible proteins.

In order to gain an insight into the possible common molecular mechanism by which the CYP2E subfamily proteins become stabilised it is necessary to look at both the normal degradative systems operating on these types of protein and the possible

molecular basis by which these normal processes could be perturbed by the presence of a substrate.

Section 4.7.1: The mechanism of Cytochrome P-450 protein turnover

The technical problems which apply to the study of the degradation of any protein apply equally to the study of P-450 turnover. The general approach to these studies is to radioactively pulse label total cellular protein synthesis and then follow the loss of the radioactive fraction of the particular protein of interest. Problems associated with such analysis *in vivo* include the possible re-use and re-incorporation of radioactive components used in the pulse distorting the apparent turnover of the protein. Attempts to block *de novo* synthesis of P-450 proteins after the pulse to reduce this possibility were hampered by the possibility that they were also blocking the production of labile proteases necessary for P-450 degradation (Ballard, 1977).

Early studies on the turnover of the whole P-450 population using pulse labelling studies suggested that there were two components in the degradation of P-450 generating a biphasic degradative profile. Firstly a fast degradative component, generating a P-450 subpopulation with a short half-life of around 7 to 8 hours, and secondly a slower degradative component generating a P-450 sub-population with a half-life of 46 to 48 hours were seen to be present (Levin & Kuntzman, 1969). It was seen that several inducing agents were capable of reducing the amount of P-450 removed by the rapid component of the degradative pathway, leaving however the contribution made by the slower component unchanged (Levin & Kuntzman, 1969; Denk & Eckestorfer, 1977; Achary *et al.*, 1980). Similar biphasic degradative pathways were noted for other endoplasmic reticulum components, for example the NADPH-cytochrome c reductase (Negishi & Omura, 1972).

Through the use of radiolabelled δ-amino levulinic acid (δALA) as a radioactive haem precursor, it was seen that haem loss from the P-450 was more rapid than turnover of the apoprotein component itself. This corroborated previous experiments which suggested that the haem component of the P-450 could be lost and exchanged between P-450 apoproteins (Farrell & Correcia, 1980. Parkinson *et al.*, 1983).

As antibodies to specific P-450 isoforms were generated it became possible to study the turnover of single P-450 isoform. Studies employing these antibodies demonstrated that different P-450 isoforms were differentially degraded and that the turnover of the total P-450 population could not be used to predict the behaviour of a particular isoform. Thus in the rat CYP1A1, CYP2B1 and CYP2B2 were seen not to possess a biphasic degradation profile and were seen only to be turned over by the slower degradative system. In contrast, CYP2A1 was seen to be degraded biphasically

generating a short half life (around 12 hours), and a long half life (around 50 hours) component to the isoforms population (Parkinson *et al.*, 1983). These observations led to the suggestion of heterogeneity in the distribution of P-450 isoforms within the endoplasmic reticulum. It was suggested that the more rapidly degraded P-450 species may be localised to portions of the endoplasmic reticulum associated with secretion and so were lost more rapidly in a more plastic environment (Chiu & Phillips, 1971). What was not clear however is how such a heterogeneity would initially be engendered to specific molecules within the same isoform population. Similar observations of a biphasic degradation were made in *in vivo* pulse labelling studies of CYP2E1 in the rat where the protein was suggested to possess both a rapid (7 hour half-life) and slow component (37 hour half-life) to its turnover pattern; addition of acetone reduced the faster turnover component contribution to CYP2E1 protein removal but left the underlying slower rate unaffected. This effect was not the result of a general reduction in the turnover of P-450 as other P-450 proteins, such as CYP2C6, showed unchanged degradation profiles in both the control and acetone treated animals (Song *et al.*, 1989).

In general terms, degradation of protein components of the endoplasmic reticulum are thought to occur via an autophagic and microautophagic lysosomal associated process and this process has been suggested to act upon the P-450 population (Ahlberg *et al.*, 1985. Masaki *et al.*, 1987). This lysosomal associated pathway is essentially viewed as a bulk flow system with the limiting factor in the rate of degradation of a particular protein being exerted by the rate of its entry into the lysosome. Once within the lysosome the protein is destroyed and no further degradative controls can operate. The microautophagic pathway is regulated by protein motifs that may be preferentially recognised by this system leading to an acceleration of degradation of specific proteins, for example pyruvate kinase and aspartate amino transferase, in certain situations such as extended starvation (Dice *et al.*, 1986; Dice *et al.*, 1987; Section 3.1).

As well as the lysosomal associated degradative system, the cell also possesses extra-lysosomal protease systems in the cytosol, mitochondria, nucleus and associated with the endoplasmic reticulum itself. In general terms these proteases tend to be larger (M_r approximately 80,000 to 500,000) than those contained within the lysosome (predominantly members of the cathepsin family, M_r approximately 20,000 to 40,000). The size disparity in these two protease classes led to the suggestion that the non-lysosomal proteases may posses regulatory domains not present in their lysosomal counterparts whose activity is regulated solely by their lysosomal containment. Some of the extra-lysosomal protease systems become activated when they recognise a protein that has been modified in some manner. An example of this is seen in ubiquitination, illustrating clearly how the cell has the capacity to regulate the amount of specific protein by employing regulatable degradative-proteolytic systems (Hershko &

Ciechanover, 1982). The extra-lysosomal proteases associated with the endoplasmic reticulum would be expected to be relevant in terms of P-450 degradation. Some of these proteases have been characterised for example Tyrase which is a small (M_r approximately 32 KDa) serine protease attached to the endoplasmic reticulum in rat hepatocytes (Saklatvala *et al.*, 1981) although it is possible that many such enzymes exist.

It was thought that haemoxygenase was responsible for endoplasmically associated P-450 degradation. Haemoxygenase is a membrane associated enzyme which catalyses the oxidative scission of haem tetrapyrole rings generating carbon monoxide, iron, and biliverdin. It was seen that induction of haemoxygenase by treatment of animals with transition metals or endotoxin also led to a decrease in the levels of P-450 (Ishizama *et al.*, 1983; Bissell & Hammaker, 1976; Maines & Kappas, 1977). It now appears however that the haem contained within the P-450 holoenzyme does not form a substrate for the haemoxygenase and so the haem must first be released from the P-450 apoprotein before it can be degraded. Inhibitors of haemoxygenase, such as selenium-protopophyrin, were also shown not to generate an induction of P-450 levels confirming this suggestion (Kappas *et al.*, 1985).

The use of primary hepatocytes was employed as a model system to study the degradation of liver proteins in general and this system has also been used to study the degradation of P-450 specifically (Sommercon *et al.*, 1981). In both primary hepatocyte cultures, and stable tissue culture lines in general, P-450 protein is lost through a process termed "gene extinction" (Steward *et al.*, 1985). This process is thought to relate to the loss of the differentiated state in the cells leading to cessation of transcription of the P-450 and a variety of other genes (Section 4.13.2). Using a rat primary hepatocyte cultures, the degradation and stabilisation against degradation, by inducing agents of a variety of P-450 species was studied; the effect of inducers on increasing the time over which a primary culture retained detectable levels of certain P-450 protein was used as an assay of these effects. CYP3A1 protein was seen to be stabilised against degradation and loss from the culture system by inclusion of tri-acetyl andomycin (TAO) in the culture medium (Watkins *et al.*, 1986), and the CYP1A subfamily were seen to be similarly stabilised in this system by the actions of polychloro-biphenyl isomers (Steward *et al.*, 1985; Pasco *et al.*, 1987; Voorman & Aust, 1988). This model system was used to show that the presence of acetone in the tissue culture medium inhibited the loss of CYP2E1 protein; the CYP2E1 protein levels were maintained at 75% of the original level of protein detected three days after the culture was established, whilst control cells grown without CYP2E1 substrates in the media lost 85% of the original CYP2E1 protein (Elliasson *et al.*, 1988).

One interpretation of these observations is that the presence of the substrate may in some manner modify the ability of the rapid degradative system to recognise the CYP2E1 protein leading to the elevation of CYP2E1 protein levels seen. It seems unlikely that a specific degradation system exists solely to remove CYP2E1 protein and so the point of regulation would be expected to reside in the CYP2E1 molecule itself rather than the protease system, which presumably also operates on other P-450 isoforms.

Section 4.7.2: Potential phosphorylational changes in CYP2E subfamily proteins as a mechanism for regulating the speed of degradation

Steric changes induced by phosphorylation are one mechanism by which a cell can modify and regulate protein components post-translationally; such modifications in a protein tertiary structure can be used to change both stability and activity. It has been a matter of speculation for many years that phosphorylational mechanisms play a part in the regulation of P-450 levels. It was seen that agents such as catecholamines, which increase the intracellular concentrations of 3', 5' adenosine mono-phosphate (cAMP) depressed the level of hepatic drug metabolism (Fouts, 1962) and membrane permeable forms of cAMP, such as N^6, O^2-dibutyrl cAMP (dbcAMP), were similarly seen to decrease the levels of P-450 in the liver (Ross, 1973). The actions of cAMP and its analogues lead to the release of the catalytically active components of the cAMP-dependent protein kinase (PK-A) from the inactive holoenzyme and these observations suggest that an increase in the activity of PK-A within a cell reduces P-450 levels.
In vitro studies investigated the phosphorylation of specific P-450 isoforms in both solublised and membrane bound preparations by monitoring the transfer of radioactive phosphate groups to the P-450 protein in the presence of purified kinase enzymes. It was seen that the rabbit CYP2B4 protein became phosphorylated at a specific site by PK-A and that this phosphorylation led to a decrease in it enzymatic activity (Pyerin *et al.*, 1983; Pyerin *et al.*, 1984; Muller *et al.*, 1985; Janzen *et al.*, 1987). Following phosphorylation, the CO-reduced spectra produced by the enzyme shifted from 450 nm towards 420nm suggesting that phosphorylation led to a change in the tertiary structure of the enzyme effecting the haem and generating an associated fall in enzymatic activity (Taniguchi *et al.*, 1982; Pyerin & Taniguchi, 1989). The specific site of phosphorylation in the rabbit CYP2B4 was analysed by peptide mapping and was seen to be present at serine 128. This serine was seen to be contained within a canonical basic kinase serine/ threonine recognition site, or "signature", arg. arg. X. X. ser. X where X represents any hydrophobic amino acid (Krebs & Beavo, 1979; Pyerin & Taniguchi, 1989). Amino-acid alignment of all the Family 2 P-450 members revealed

the presence of this kinase signature in most of the proteins; in some members of the CYP2C subfamily, it is seen that the serine has become replaced by a threonine and in one chicken Family 2 sequence by an arginine. This signature is lost in other P-450 families although the arg. arg. portion of the signature is retained; in Family 1, although the signature is present it has become interrupted by an additional 8 amino acids (Nelson & Strobel, 1988).

In vitro studies on a variety of different P-450 using both "basic" (i.e. those which recognise sites flanked by groups of basic amino acids, such as PK-A, and the calcium/phospholipid dependent kinase PK-C) and "acidic" (recognising sites flanked by acidic amino acid residues, such as Casein kinase II) kinases supported the observation that the main site of phosphorylation in the Family 2 P-450 was serine 128/129 and was mediated by basic kinases; no phosphorylation resulted from the action of acidic kinases. Certain members of the CYP2C family were not phosphorylated by PK-A, although it is not possible, for nomenclature reasons, to assign which specific CYP2C isoforms these proteins represent. This observation may relate to the replacement of serine 128 by threonine as seen in some CYP2C subfamily isoforms for example. Members of Families other than Family 2 were also seen to be phosphorylated by PK-C suggesting that sites other than the serine 128/129 may be phosphorylated; other phosphorylation sites have been observed in some classes of P-450 involved in steroid biosynthesis, for example serine 267 in the human CYP19 aromatase family was shown to be a target for phosphorylation by PK-A (Pyerin & Taniguchi, 1992). PK-C was seen to phosphorylate some P-450 isoforms but this was form-specific, for example rat CYP2B1 and CYP2B2 were both phosphorylated by PK-A whereas only CYP2B1 was phosphorylated by PK-C (Pyerin *et al.*, 1987). Treatment of primary rat hepatocytes with the calcium ionophore A23187, however, did not lead to any phosphorylation of either CYP2B1 or CYP2B2 suggesting that PK-C, and thus calcium signalling pathways, may not produce any effect on these isoforms (Koch and Waxman, 1989).

Experiments performed on a series of P-450 isoforms in rats suggest that the changes seen to occur *in vitro* may also control P-450 *in vivo*. Members of the CYP2B subfamily were induced by phenobarbital and the effects of treatments known to modify intracellular cAMP levels on CYP2B isoforms was studied *in vivo*. Treatment of the animals with dbcAMP (a cAMP anologue) and glucagon (which increases intracellular cAMP levels) elevated CYP2B1 and CYP2B2 phosphorylation suggesting that PK-A plays an *in vivo* role in this process (Koch and Waxman, 1989).

From these studies it is possible that phosphorylational changes at the kinase signature around serine 128/129 in Family 2 P-450 can form part of the post-translational control

mechanism by which a cell can change the structure, activity and possibly stability of a P-450 protein.

Section 4.7.3: The nature of the Family 2 serine 128/129 phosphorylation site

From amino acid alignment it was seen that most family 2 P-450s contained a canonical basic kinase signature around a phosphorylatable serine 128/129 residue. This area of the P-450 protein sequences is highly conserved suggesting that it may be part of a common structural element (Figure 4.7). The conserved features in this region include the retention of basic amino-acids (either arginine or lysine) in the arg. arg region of the Family 2 kinase signature in all P-450 sequences, both mammalian and bacterial as well as a tryptophan residue a few residues towards the N-terminus from this basic region (Nelson & Strobel, 1988).

Using the computer generated alignment made between the available CYP2E subfamily predicted amino-acid sequences and the crystallographically solved bacterial P-450cam (CYP101) it can be seen that all the conserved elements in the kinase signature would be predicted to reside within the C-helix by analogy to the P-450cam structure (Figure 5.5). In P-450cam arg 112 and the nearby glu 108, both residues of the C-helix, are seen to form hydrogen bonds with the haem proprionate group (Poulos *et al.*, 1987); on alignment it can be seen that these amino-acids are represented by the arginine residue of the kinase signature and the conserved tryptophan (Figure 4.7). Thus the phosphorylatable serine of Family 2 appears to be located within a highly conserved region of the P-450 structure which may contribute to the C-helix and is therefore likely to be involved in the interaction between the apoprotein and the haem group. The introduction to this evolutionarily conserved basic region of the P-450 protein of two negative charges as the result of phosphorylation may be expected to interfere with the charge of the protein and potentially the interaction between the C-helix and the haem.

It is of interest that, although no comparable phosphorylation site is present within the C-helix region of P450cam, a search of the P450cam protein sequence for the basic kinase signature, using the GCG package Best program, revealed a site (arg. arg. phe. ser 293. leu) just N-terminal to the helix-K (Figure 5.5). The K-helix region is another area of the P-450cam protein seen to interact with the haem group, particularly val 295 and arg 299 (Poulos *et al.*, 1987; Section 5.3.2). If a phosphorylation event is possible at this site in the bacteria, a perturbation of the haem environment may result.

In this manner, phosphorylation may be a mechanism by which the cell could exert post-translational control over P-450 enzymes. Phosphorylation may lead to a change in structure in the apoprotein and potentially change the apoprotein:haem interactions, as suggested by spectrophotometric analysis in the rabbit CYP2B subfamily following

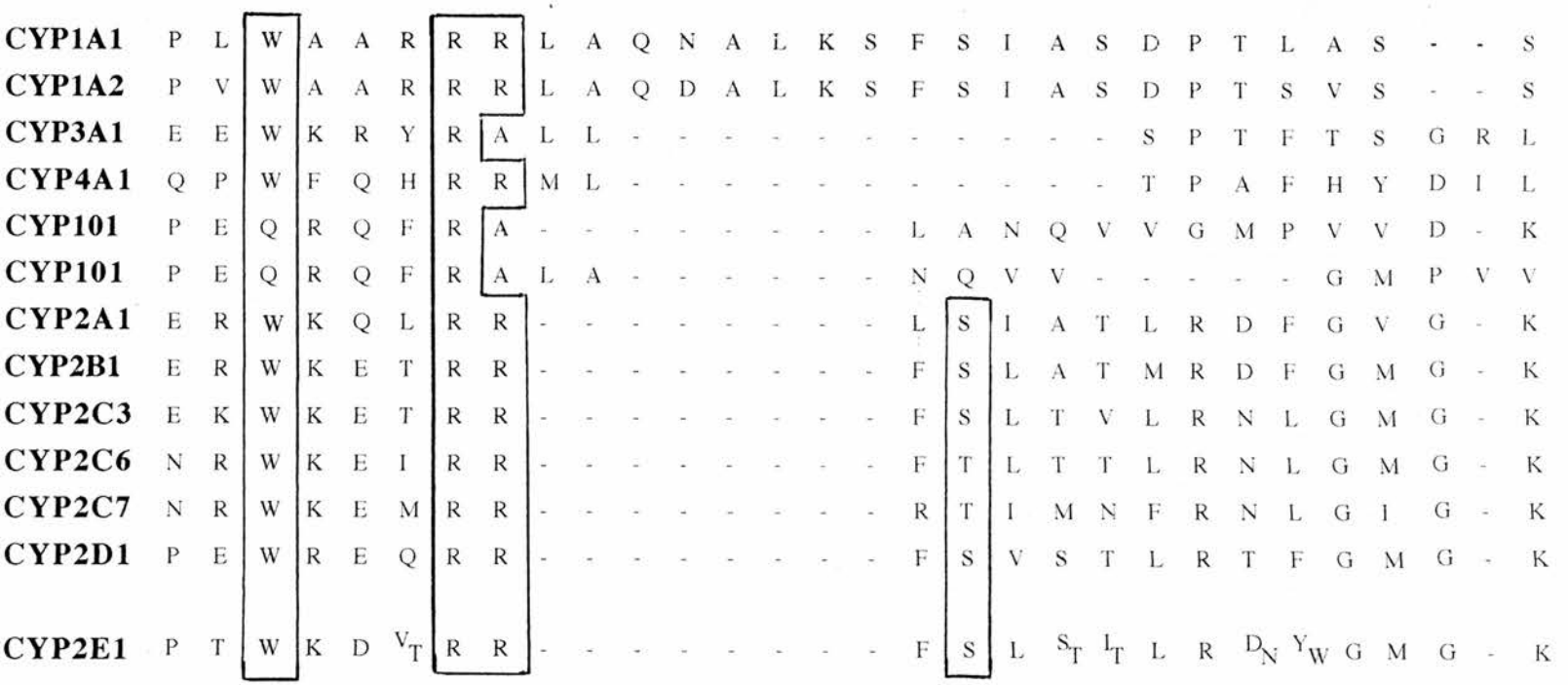


Figure 4.7: An alignment of the predicted C-helix regions of several rat xenobiotic metabolising P-450 protein sequences and the CYP2E subfamily predicted C-helix consensus sequence (See Figure 5.5) with the C-helix of CYP101 (P-450cam, Poulos *et al.* , 1987). The potential serine phosphorylation site, basic-kinase arginine recognition motif and completely conserved tryptophan residues are boxed. The sequences were taken from a compilation of mammalian P-450 sequences generated by Nelson and Strobel (1988); the upper and lower alignment possibility for the mammalian sequences relative to CYP101 were taken from Nelson and Strobel (1988) and Zvelebil *et al.* (1991) respectively.

phosphorylation (Taniguchi, *et al.*, 1982; Pyerin & Taniguchi, 1989). Phosphorylation may also form a signal for degradation of the CYP2E subfamily by a rapid proteolytic system as a result of structural modifications.

Section 4.8: A potential role of serine 129 phosphorylation in the post-translational control of CYP2E subfamily protein

Purified rat CYP2E1 protein was phosphorylated by PK-A and this event produced a shift in the carbon monoxide-reduced spectra from 450nm towards 420nm. In common with similar studies on other Family 2 isoforms, tryptic analysis of the CYP2E1 protein showed that phosphorylation occurred at serine 129 (Eliasson *et al.*, 1990).
The level of serine 129 phosphorylation was seen to be elevated in rat primary hepatocyte cultures by the presence of 8-bromoadenosine (similar in action to the membrane permeable cAMP anologue dbcAMP), glucagon and adrenalin, both of which elevate the level of intracellular cAMP, for example the presence of glucagon increased the control CYP2E1 phosphorylation level of 27 arbitrary units to 100 (Eliasson *et al.*, 1990). The loss of CYP2E1 protein from the primary hepatocyte cultures was seen to be elevated by the inclusion of the agents capable of increasing CYP2E1 phosphorylation; the loss of CYP2E1 from 2 day old cultures was increased by 40 to 50% by dbcAMP addition for example (Eliasson *et al.*, 1990). It was seen that inhibitors of the autophagasomal/lysosomal degradative pathway, such as NH_4Cl, leupeptin, chloroquine and 3-methyladenine, did not inhibit the increased degradation of CYP2E1 on addition of glucagon and it was suggested that the accelerated degradation resulted instead from a Mg^{2+}-ATP activated proteolytic system associated with the endoplasmic reticulum (Ingelman-Sundberg *et al.*, 1992). The rate of loss of CYP2E1 protein from the primary hepatocytes was seen to be decreased by the addition of cycloheximide suggesting that components of the degradation system were labile and needed continual synthesis (Eliasson *et al.*, 1988; Ingelman-Sundberg *et al.*, 1992).
As was noted in previous experiments (Eliasson *et al.*, 1988) the inclusion of CYP2E1 substrates in the culture media reduced both the level of phosphorylation and the increased loss of the CYP2E1 protein produced on addition of the cAMP elevating agents. The level of phosphorylation, presented as a percentage of the control value without the substrate, was seen to be 53%, 61% and 39% in the presence of isopropanol, ethanol, and acetone respectively. The presence of the substrate also blocked the carbon monoxide-reduced spectral shift towards 420nm in studies on purified proteins in association with PK-A (Eliasson et al., 1990). Treatment of the primary hepatocyte cells with insulin which, amongst other things, decreases the intracellular levels of cAMP, led to stabilisation of the CYP2E1 protein; in the absence

of any substrate in the media a doubling in the amount of retained CYP2E1 protein was seen on addition of insulin (Johansson *et al.*, 1989). All these effects were suggested to be specific to the regulation of CYP2E1 phosphorylation and degradation alone as no change in the rate CYP2B1 phosphorylation was seen (Eliasson *et al.*, 1990; Johansson *et al.*, 1989).

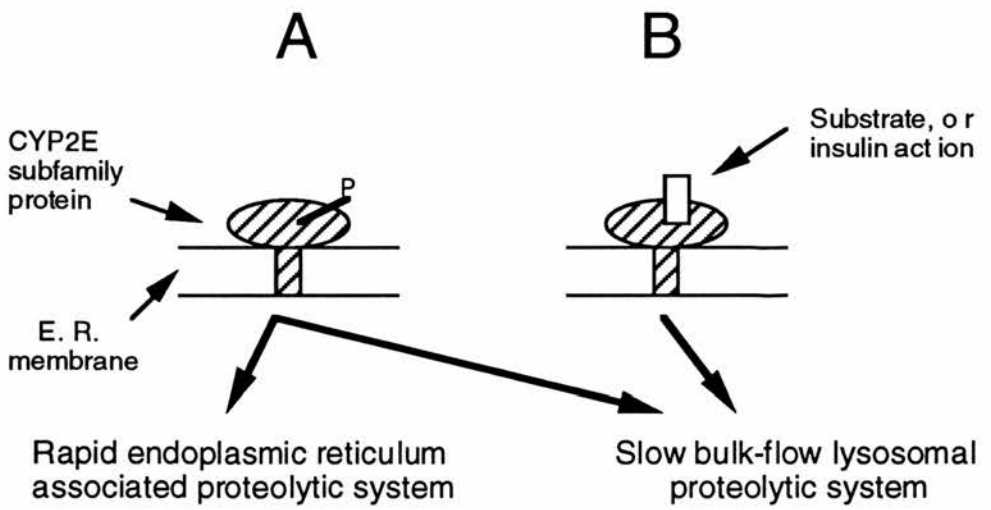
In an *in vivo* study of CYP2E1 degradation pathways in intact rat livers, it was seen that feeding the animals with CYP2E1 substrates led to, as well as the increase in CYP2E1 protein levels, an increase in the presence of CYP2E1 degradation products within lysosomes suggesting a shift in the pathway by which the CYP2E1 protein becomes degraded following substrate stabilisation (Ronis *et al.*, 1991).

Taken together, and in connection with the observed biphasic pattern of CYP2E1 degradation (Song *et al.*, 1989), these pieces of information suggest that there are potentially two degradative systems operating on CYP2E1. The rapid component, generating a CYP2E1 half- life of 7 hours, may be associated with the endoplasmic reticulum and the slower component, generating a CYP2E1 half-life of 37 hours, may be associated with the lysosomal degradative processes. These results also suggest that the rapid proteolytic pathway acts upon CYP2E1 protein after serine 129 becomes phosphorylated and that agents, such as dbcAMP, adrenalin and glucagon accelerate both the phosphorylation and the degradation of CYP2E1 proteins through this pathway; inhibitors of the slower autophagasomal pathway do not affect the acceleration of CYP2E1 degradation by these agents. Phosphorylation may potentially lead to a conformational change within the CYP2E1 protein and modification of the haem interactions, either or both of which events may enable the rapid proteolytic system to degrade the protein. The presence of CYP2E1 substrates, or the blocking of the phosphorylation pathway through the action of cAMP agonists such as insulin, may reduce phosphorylation, blocking the structural change and the CYP2E1 recognition by the rapid proteolytic system. This phosphorylation block may then switch the method of CYP2E1 removal instead predominantly to the slower, bulk-flow, lysosomal system as suggested by the increased levels of CYP2E1 degradation products in the lysosomes following chemical induction (Ronis *et al.*, 1991).

In vitro studies suggest that cellular signalling systems associated with glucagon lead to an acceleration of CYP2E subfamily protein phosphorylation and degradation, whereas in contrast, insulin appears to stabilise the protein. This does not fit with the apparent control mechanisms seen to operate on the protein *in vivo*. In the diabetic state, insulin levels fall as the β-cells become destroyed, and glucagon levels rise as the body switches from the use of glucose to fatty-acids as a fuel source (Johnston *et al.*, 1984. Pilkis *et al.*, 1988). In the diabetic state the level of both CYP2E1 protein and mRNA are seen to be elevated and this elevation is seen to be reversed by the

administration of insulin (Section 3.4.1). It may be expected from this *in vivo* observation that lowered insulin levels, and presumed glucagon raised levels, would increase the levels of CYP2E1 protein; this is in stark contrast to the observed effects of these hormones on CYP2E1 protein in tissue culture systems where glucagon is associated with accelerated CYP2E1 degradation. By producing the effects suggested by the *in vitro* experiments, hormonal signalling may generate a more responsive CYP2E1 protein induction system given the proposed endogenous role of the CYP2E subfamily proteins as gluconeogenic enzymes. Thus in a starved or diabetic state where glucagon levels are high it would be expected that the rate of CYP2E1 phosphorylation and so degradation would be accelerated, but increased acetone levels in this situation prevents phosphorylation from occurring and so the CYP2E1 protein is stabilised. In the non-starved state the levels of acetone fall and the CYP2E1 protein becomes available to phosphorylation potentially leading to accelerated degradation but the fall in glucagon and rise in insulin levels reduce the activity of the phosphorylating system and the CYP2E1 loss. In this manner antagonistic actions mediated by the biochemical and hormonal changes associated with the normal, starved and diabetic states may allow a more rapidly controlled regulation of CYP2E subfamily protein levels to be mediated and acetone-derived inductions of CYP2E protein can be both rapidly generated and quickly disapated. These ideas are represented in Figure 4.8.

Figure 4.8: The proposed role of serine 129 phosphorylation in the control of the CYP2E subfamily protein stability. **A:** In the absence of a substrate serine 129 is available for phosphorylation, the level of phosphorylation is accelerated by an elevation in cAMP levels resulting from the actions of hormones such as glucagon. The CYP2E subfamily protein is available, as a result of structural changes induced by serine 129 phosphorylation, to both a rapid endoplasmic reticulum-associated, and lysosomal degradation systems. **B:** In the presence of a substrate, or if cAMP levels are decreased by the actions of hormones such as insulin, the level of serine 129 phosphorylation falls. The CYP2E subfamily protein is stabilised in these instances as they are only degraded by the slower, lysosomal proteolytic system.



Section 4.9: The potential role of serine 129 in the control of mouse Cyp2e1 protein degradation

By analogy to the studies on the rat CYP2E1 in primary hepatocytes (Eliasson *et al.*, 1990; Johansson *et al.*, 1989; Ingelman-Sundberg *et al.*, 1992) serine 129 in the mouse Cyp2e1 would be expected to be phosphorylated by PK-A and may influence the rate of removal of the protein by a putative endoplasmic reticulum-associated proteolytic degradation system. By employing Cyp2e1 cDNA clones in which the serine 129 had been mutated, the effect of the change at this site on the accumulation of heterologously expressed Cyp2e1 protein was monitored in mammalian tissue culture using a transient expression system.

Section 4.9.1: Generation of Cyp2e1 serine 129 mutations

The serine 129 within a mouse Cyp2e1 cDNA was modified by site-directed mutagenesis to alanine 129 and glycine 129 in two separate constructs. Alanine and glycine were selected to replace serine as they most closely resemble serine in size, structure and charge and so would not be expected to peturb greatly the folding of the mutant Cyp2e1 protein; these three amino acids are all classified together based on a previously established mutational matrix (Taylor, 1986). The area of the P-450 sequence containing serine 129 is highly conserved and expected to form the C-helix by analogy to P-450cam (Figure 4.7 and Figure 5.5). It is of note, however, that the serine at this site is replaced by an arginine, with its much larger R-group, and threonine in a chicken and rabbit CYP2C protein (Nelson & Strobel, 1988) suggesting that any slight steric perturbations introduced by these mutagenic changes may be accommodated by this region of the P-450 structure.

The mouse full-length mouse Cyp2e1 cDNA was subcloned into the *Xba* I and *Sal* I restriction endonuclease sites of the bacteriophage M13mp18. Single-stranded M13mp18 DNA generated from Cyp2e1 cDNA recombinant plaques was prepared from the *E. coli* JM109 host cells and this DNA was used both to transform BW313, a *dut⁻ ung⁻* strain of *E. coli* , and as a template in sequencing reaction to check that the mutagenic oligonucleotides annealed in the expected position within the Cyp2e1 cDNA. Single-stranded M13 DNA was prepared from the BW313 *E. coli* and this DNA was used in the mutagenic reactions. The *dut⁻ ung⁻ E. coli* strain contains elevated intracellular levels of dUTP (*dut*) and uracil glcosylase (*ung*) which increases the incorporation of uracil into DNA templates in a few instances following replication in place of a thymidine residue in the original template. Using single-stranded templates containing several uracil residues however generates a second strand with thymidines in the place of the randomly added uracils.The products of this reaction are then used to transform non-*dut⁻ung⁻ E. Coli* strain which inactivates the original uracil containing strand and so only the second thymidine containing and potentially mutated strand is replicated (Kunkel *et al.*, 1985). Mutated single-stranded M13 DNA prepared in this manner from several plaques was sequenced and the introduction of the desired mutations confirmed; replicative form double stranded DNA was prepared from successfully mutated M13 plaques. The two mutant Cyp2e1 cDNA clones were then subcloned into the *Kpn* I and *Hind* III restriction endonuclease sites of the transient eucaryotic expression vector pCMV4. Both mutant clones were then resequenced in the pCMV4 vector by double-stranded sequencing to confirm the presence of the mutations in the final constructs. The introduction into the pCMV4 vector of the various portions of poly-linkers from the pBS SK II (-) and M13mp18 vectors in the preparation of

these mutated cDNA constructs allowed the use of the *Not* I restriction endonuclease site to clone in the unmodified Cyp2e1 cDNA directly. The orientation of the Cyp2e1 cDNA cloned into the *Not* I site was assayed by the use of diagnostic *Sca* I restriction endonuclease sites and these constructs were sequenced from the pCMV4 double-stranded template to confirm the presence of the serine 129 codon. The oligo-nucleotides employed, the determination of the mutations and the strategy used in the preparation of these normal and mutant Cyp2e1 constructs is shown in Figures 4.9 and 4.10.

Section 4.9.2: The pCMV4 vector expression system and the proposed model for P-450 degradation generated through the use of the mutated Cyp2e1 proteins in COS 7 cells

The pCMV expression system vectors contain the powerful (-730 to +3) promoter region of the human cytomegalovirus (CMV, Towne strain) major intermediate early gene as well as the SV40 and f1 bacteriophage origins of replication to allow replication in both mammalian and bacterial cells. A synthetic fragment of DNA corresponding to the 5' UTR of the Alfalfa-mosaic virus 4 RNA is also placed downstream of the CMV promoter which acts as a translational enhancer by decreasing the translational initiation requirements of the generated message.The transcripts generated from the CMV promoter are terminated by a poly-A addition signal present in a human growth hormone 3' UTR region present downstream. The combined vector has proved a powerful tool in the generation of heterologous protein expression in Simian COS cell tissue culture systems (Anderson *et al.*, 1989).

The PCMV4 constructs, although they contain an SV40 origin of replication, are only present transiently within mammalian cells following transfection and do not integrate into the genome of the recipient cells. Thus the heterologous protein encoded by the construct is expressed only during the time in which the CMV construct is present within the cell, and as the plasmid is lost then both the heterologous transcript and protein cease to be produced and are degraded. The Simian COS 7 stable cell line is derived from the kidney and in common with most other stable mammalian cell lines does not retain expression of several P-450 genes; as a result the cell line would not be expected to contain a complicating endogenous CYP2E1 protein. By using transiently generated expression of parental and mutant Cyp2e1 protein forms in the COS 7 cells it was intended to generate a system mimicking the event of CYP2E1 gene extinction and protein loss studied previously in primary hepatocytes (Eliasson *et al.*, 1990; Johanson *et al.*, 1989). Using this system the role of serine 129 as a phosphorylation site in the regulation of Cyp2e1 protein degradation could then be investigated.

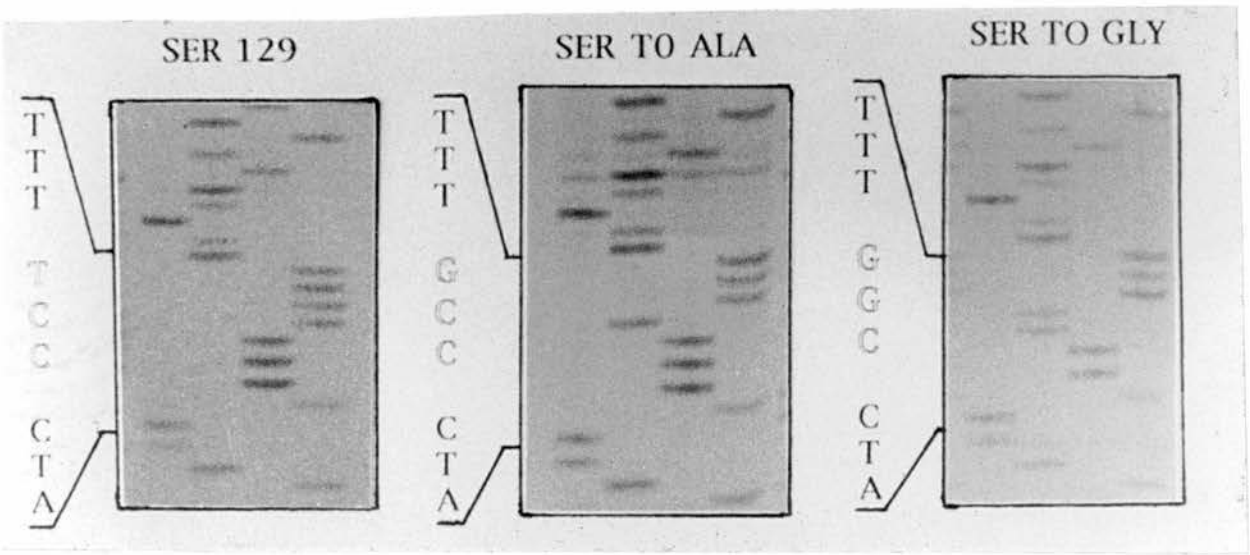


Figure 4.9: The generation of Cyp2e1 cDNA sequences with directed mutations in serine 129. Serine 129 in the parental Cyp2e1 cDNA was mutated to encode alanine and glycine through the use of oligonucleotide-directed mutagenesis. The mutations were confirmed by sequencing of M13 single-stranded DNA templates and the cDNA sequences subcloned into the pCMV4 mammalian transient expression vector. The figure shows the confirmation of the presence, and absence, of the serine 129 codon in the native and 2 mutant pCMV4 constructs respectively through the use of the final pCMV4 constructs as double-stranded DNA templates for sequencing. The mutated serine codon is shown in hollow type. **SER 129:** The parental Cyp2e1 cDNA containing the serine 129 codon. **SER TO ALA:** The mutated Cyp2e1 cDNA encoding alanine at posistion 129. **SER TO GLY:** The mutated Cyp2e1 cDNA encoding glycine at posistion 129. The tracks are loaded, left to right, A, G, C, T.

The 2 mutagenic oligonucleotides employed:

1. Alanine 129: GGATACTTAGGG**C**AAACCTCCGC
2. Glycine 129: GGATACTTAGG**CC**AAACCTCCGC
   (Parental 129: GGATACTTAGGGAAAACCTCCGC)

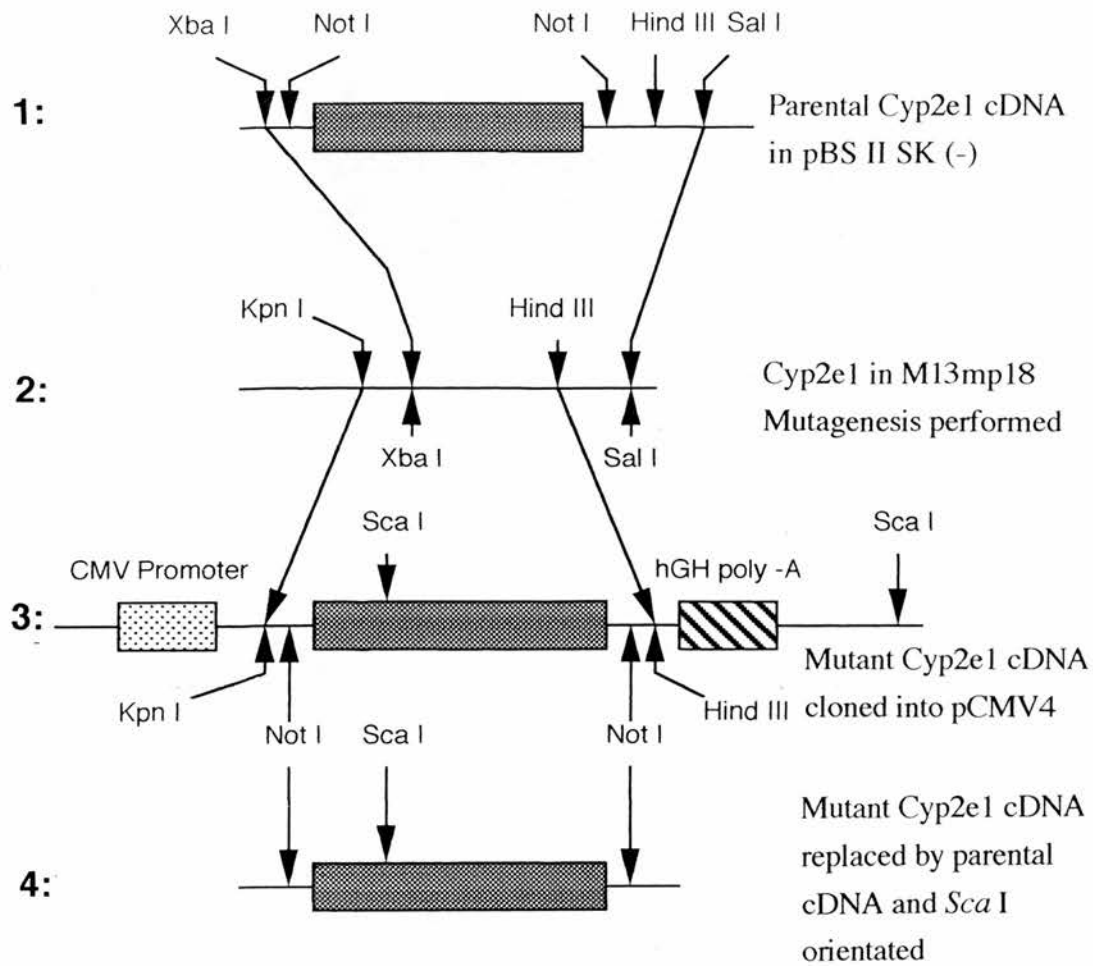


Figure 4.10: The cloning strategy employed to generate the 3 Cyp2e1 cDNA constructs in the pCMV4 mammalian transient expression vector. Through the use of the available restriction endonuclease sites in the pBS SK II (-) and M13mp18 vectors (**1** & **2**) the oligonucleotide-directed Cyp2e1 cDNA mutants were directionally cloned into the *Kpn* I and *Hind* III restriction sites in the pCMV4 mammalian transient expression vector (**3**). The parental Cyp2e1 cDNA was inserted into the *Not* I restriction endonuclease sites introduced into the pCMV4 vector as a result of these manipulations and the orientation of the clones determined through the use of a diagnostic *Sca* I digestion (**4**). **CMV Promoter:** The human cytomegalovirus (Towne strain) major intermediate early gene promoter region. **hGH poly-A:** The poly-(A) addition site of the human growth hormone gene.

The same predicted processes leading to substrate-induced stabilisation of the CYP2E1 protein in the primary hepatocytes would be expected to occur in kidney cells; the Cyp2e1 protein, for example, was seen to be elevated on acetone treatment in the mouse kidney (Figure 4.2).

By replacing the serine 129 in the Cyp2e1 mutants with similar but non-phosphorylatable amino acid residues it would be expected that the mutant Cyp2e1 proteins should behave as if they were constantly in the presence of a substrate. As the mutant forms of the Cyp2e1 protein cannot be phosphorylated at position 129 they would not be expected to be degraded by the postulated, rapid, endoplasmic reticulum-associated proteolytic system but instead being removed only by the slower, potentially bulk-flow, lysosome-associated degradation mechanism. By contrast the normal form of the Cyp2e1 protein, with a serine 129 phosphorylation site still present, would be expected to be removed by both the rapid and slow degradative pathways. After the CMV vector has been lost from the transfected cells the differences in the pathways available to the cell to remove the normal and mutant heterologous Cyp2e1 proteins should manifest themselves. The normal Cyp2e1 protein, degraded by both the rapid and slow removal pathways, should be observed to be lost from the system more rapidly than the mutant forms acted upon theoretically only by the slower degradative pathway. By contrast the mutant proteins may be expected to accumulate in the cell as they are only removed by the slower lysosomal degradative pathway. It should therefore be possible to determine the differences in the rates of degradation of the different proteins, by a combination of the length of time which they are retained in the cell line after loss of the CMV construct and an increase in mutant protein levels relative to those of the native protein due to an accumulation of the mutant forms. The transient expression of Cyp2e1 protein itself acts as an easily assayed protein pulse without requiring the need to use any other labelling approaches as the recipient COS 7 cell line would not be expected to contain any complicating endogenous CYP2E1 protein.

Section 4.9.3: Transfection of Cyp2e1 pCMV4 constructs and the time course for Cyp2e1 protein loss from COS 7 cells

The ability of the pCMV4 construct to produce immunologically detectable levels of Cyp2e1 protein in COS 7 cells was established; COS 7 cells were transfected with the normal Cyp2e1 cDNA pCMV4 construct contained in both the correct and reverse orientation for expression of the Cyp2e1 transcript from the CMV promoter (Figure 4.11). The cells were harvested two days after transfection, disrupted by sonication and total cellular protein was probed with polyclonal antisera raised to purified rat CYP2E1 protein.

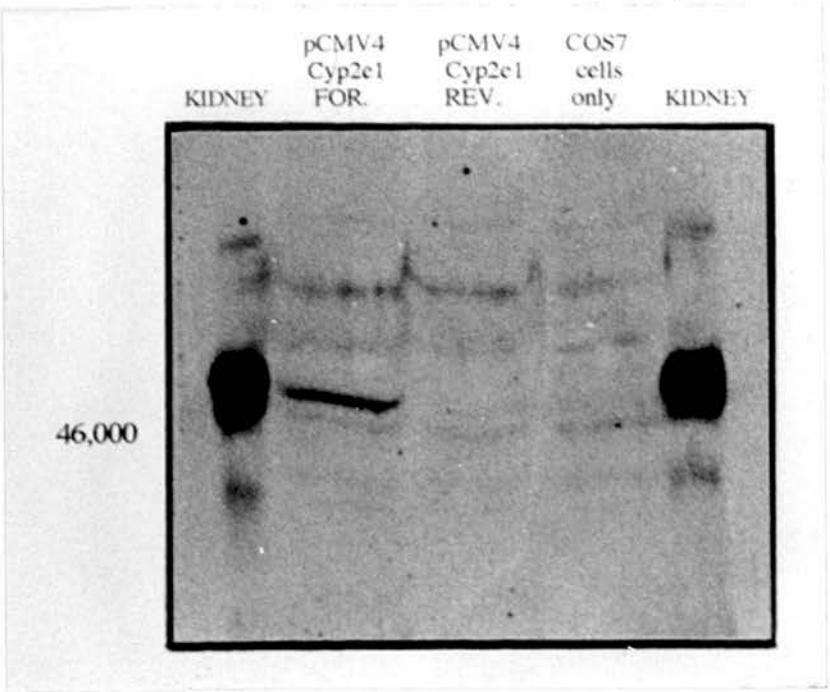


Figure 4.11: The expression of Cyp2e1 protein in COS 7 cells directed by the pCMV4 transient espression construct containing the parental Cyp2e1 cDNA. COS 7 cells were transfected with the pCMV4/parental Cyp2e1 cDNA construct in the correct orientation for protein generation (**pCMV4 Cyp2e1 FOR.**), the pCMV4/parental Cyp2e1 cDNA construct in the reverse orientation for protein generation (**pCMV4 Cyp2e1 REV.**, see Figure 4.10), and mock transfected without any DNA (**COS7 cells only**). Total COS 7 protein was isolated and 50μg of each sample separated by SDS/PAGE on a 12% gel together with 20μg of DBA2/N male mouse kidney microsomes (**KIDNEY**). The protein was transferred to a nitrocellulose filter and probed with polyclonal antisera raised to purified rat CYP2E1 protein; the immune-complex was visualised using the enhanced chemiluminescence technique (Section 2.21.3).The Cyp2e1 protein migrated with a M_r of approximately 53,000 and no bands other than those shown were detected.

Cyp2e1 protein was seen to be produced from the correctly orientated Cyp2e1 construct but no bands were present in protein prepared from cells either transfected with reverse orientated Cyp2e1 cDNA, or mock transfected without DNA. These results demonstrate that the pCMV4 vector produces immunodetectable levels of Cyp2e1 protein and that there is no complicating endogenous CYP2E1 protein in the COS 7 cell line (Figure 4.11).

The Cyp2e1 pCMV4 construct was transfected into a series of COS 7 cultures; a transfected culture was harvested, sonicated to disrupt the cells and total cellular protein prepared at daily intervals over an eight day period. The transfected cells were washed and re-fed with fresh media every two days following the transfection but were not split; total cellular protein from each time point was probed using antisera raised to purified CYP2E1 protein. It was seen that Cyp2e1 protein was expressed at detectable levels up until day six of culturing but was then lost. The viability of the COS 7 cells was seen to be little changed between day six and seven of culturing through the use of a Nigrosin dye exclusion assay; the cells were 90% and 85% viable at day six and day seven respectively.

Section 4.9.4: The effect of the alanine and glycine 129 mutant Cyp2e1 proteins on the level of accumulation and rate of loss of the Cyp2e1 protein

Separate COS 7 cultures were transfected with equal amounts of the two mutant Cyp2e1 and normal Cyp2e1 PCMV4 constructs. Total cellular protein was harvested from the cultures daily over an eight day period, the time period during and beyond which normal Cyp2e1 protein expression had been detected in this system. Despite the lack of serine 129 in the two mutant Cyp2e1 protein forms all the Cyp2e1 proteins, both mutant and normal, were seen to be lost from the COS 7 cellular protein at day six; these observation were repeated in three separate experiments. The cellular proteins obtained from the staged cell extracts were probed using both ^{125}I-protein-A and Enhanced Chemi-luminescence (ECL, Amersham) detection systems but neither technique detected the presence of Cyp2e1 protein beyond day six of culturing after transfection. The extracts also demonstrated that the relative levels of mutant and native Cyp2e1 protein present at each sample point in the expression study were not markedly different; there was no apparent accumulation of the mutant protein relative to the native protein (Figure 4.12).

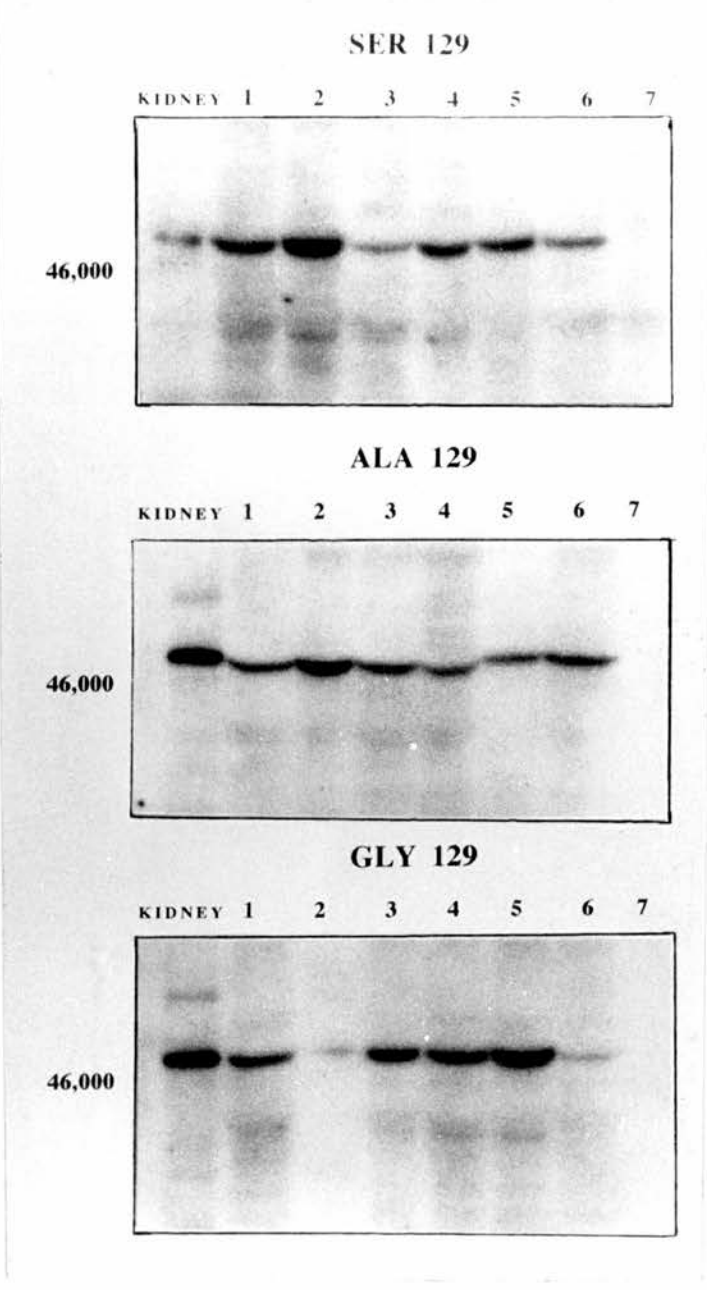


Figure 4.12: The effect of the removal of serine 129 on the accumulation and retention of the Cyp2e1 protein in COS7 cells using the pCMV4 transient expression vector. COS 7 cells were transfected with the parental, serine 129 containing, Cyp2e1/pCMV4 construct (**SER 129**), and the two mutant Cyp2e1 cDNA pCMV4 constructs containing alanine at posistion 129 (**ALA 129**), and glycine at posistion 129 (**GLY 129**). Total COS 7 protein was isolated from cultures maintained for 1 to 7 days following transfection (**1** to **7**) and 100μg of each sample was separated by SDS/PAGE on 12% gels together with 10μg of DBA2/N male mouse kidney microsomes (**KIDNEY**). The protein was transferred to nitrocellulose filters and probed with polyclonal antisera raised to purified rat CYP2E1 protein; the immune-complex was visualised using ^{125}I-labelled protein A. The Cyp2e1 protein migrated with a M_r of approximately 53,000 and no bands other than those shown were detected.

Section 4.9.5: The effect of insulin on the Cyp2e1 protein levels

In the primary hepatocyte experiments the addition of insulin to the cells was reported to increase the amount of protein retained in the cells and this may result from the lowering of intracellular cAMP levels as a result the actions of insulin (Johansson *et al.*, 1989). The effect of insulin on the protein levels of both the native and mutant Cyp2e1 at day four after transfection was studied; the cells were treated identically except one group had insulin present in the media (1×10^{-7}M). It would be expected that the presence of insulin would lead to a blocking of the degradation of the native Cyp2e1 protein and so an elevation, due to an accumulation, of protein levels in this cell population, relative to the control cells, would be seen. By contrast he relative protein levels of the mutant Cyp2e1 proteins would be expected to be unaffected by the presence or absence of insulin.

COS 7 cells were transfected with the contructs in the presence, or absence of insulin and were harvested 2 days after transfection. The COS 7 cells responded to the presence of insulin in the media by an increased protein concentration present in the cell extracts relative to the control cells suggesting an increase in protein synthesis and potentially proliferation as noted previously (Section 3.3.4). Following normalisation of the protein concentrations in the samples it was seen that the presence of insulin in the media appeared to lead to a slight elevation in the levels of all the Cyp2e1 protein generated by both the native and mutant constructs alike (Figure 4.13).

This observation does not support a role for a serine 129 phosphorylation blocking affect of insulin in its presumed role as a stabilising agent of the CYP2E subfamily proteins. This suggests either that insulin may be stabilising the CYP2E subfamily proteins by a post-translational change elsewhere in the protein, and present in both native and mutant Cyp2e1 proteins, or as a result of non-specific global changes in the rate of translation of a whole series of transcripts due to changes produced in the translational machinery (Section 3.3.4).Similar observations have been made in studies on the effect of insulin on steroid hormone metabolising P-450 in primary culture; here it was noted that insulin generated an overall increase in the metabolic activity of these P-450 (Gulati & Skett, 1989). The cellular response to insulin also leads to a slowing of membrane bulk-flow. Insulin treatment, in certain cell types, leads to the appearance of elevated levels of glucose transporter proteins and these actions may be mediated through either a slowing of endocytosis or an acceleration of exocytosis; increases in the levels of other receptors, such as the transferrin receptor, have also been noted (James *et al.*, 1988; Tarner & Lienhard, 1987). It is another possibility therefore, that the increased levels of Cyp2e1 protein following addition of insulin, may relate to a decrease activity in the presumed bulk-flow lysosomal degradative pathway.

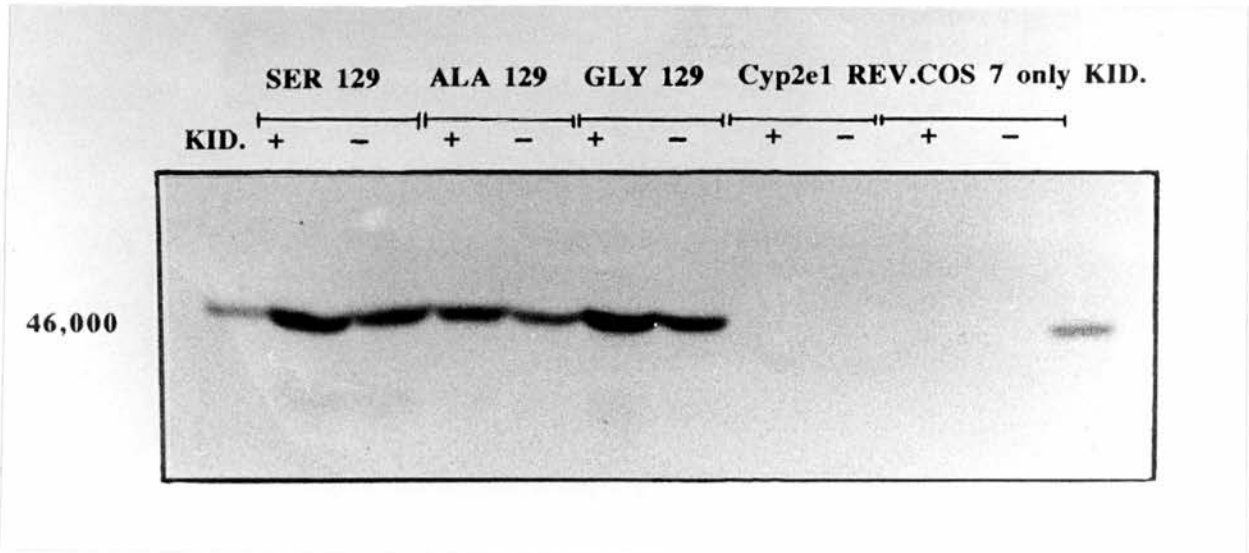


Figure 4.13: The effect of the presence of insulin in the culture medium on the level of mutant and parental Cyp2e1 protein generated in COS 7 cells by the pCMV4 transient expression constructs. COS 7 cells were transfected with the parental Cyp2e1/pCMV4 construct in the correct orientation (**SER 129**), the 2 mutant Cyp2e1 pCMV4 constructs with alanine (**ALA 129**) and glycine (**GLY 129**) at amino acid posistion 129 respectively, the parental Cyp2e1 cDNA in the reverse orientation (**Cyp2e1 REV.**) and without DNA (**COS 7 only**), in the presence (+), or absence (-) of 1 X 10^{-7} M insulin in the medium. Total cell protein was isolated 2 days after tranfection, and 100μg of each sample was separated by SDS/PAGE on a 12% gel together with 20μg of acetone-treated female DBA2/N mouse kidney microsomes (**KIDNEY**). The protein was transferred to a nitrocellulose filter and probed with polyclonal antisera raised to purified rat CYP2E1 protein; the immune-complex was visualised using ^{125}I-labelled protein A. The Cyp2e1 protein migrated with a M_r of approximately 53,000 and no bands other than those shown were detected.

Section 4.9.6: Possible explanations for the lack of an observed difference between the behaviour of the mutant Cyp2e1 proteins and the native Cyp2e1 in the COS 7 system

According to the postulated mechanism by which the relative contribution made by the two degradative mechanisms proposed to operate on CYP2E subfamily protein are controlled, the removal of the serine 129 in the Cyp2e1 mutants should present the cell with a much more stable Cyp2e1 protein. From the experimental system employed in these experiments it does not appear that this is the case. Both mutant and normal Cyp2e1 protein is lost from the cells by the same time point, accumulates to comparable levels over the period of expression and are equally affected by the presence of insulin in the medium.

This observation could be explained by a variety of possibilities. It is possible that the postulated labile endoplasmic reticulum-associated protease system suggested to mediate the rapid and regulatable degradation of the CYP2E subfamily protein (Ingelman-Sundberg *et al.*, 1992) may be subject to gene extinction and loss from the stable tissue culture line in a manner similar to that observed for P-450 proteins themselves. However the levels of Cyp2e1 protein were not seen to accumulate over the period of expression suggesting that the lysosomal/ autophagasomal degradative pathways were still operational.

Alternatively is possible that the phosphorylation site at serine 129 does not play a role in the regulation of stability of the CYP2E subfamily proteins mediated by the presence of substrates. The observed elevation of phosphorylation at this site may therefore represent a marker of a change produced by the absence of the substrate and be associated with, rather than the cause of, an accelerated degradation. The cause for the slowing of degradation by the presence of the substrate may therefore relate to a change within another potential structural signal, allowing degradation by a rapid proteolytic system to occur. It has been noted in the purification of several P-450s, other than those from Family 2, that addition of an appropriate substrate to the purification procedure increases the yield of the resultant active P-450 protein. This observation suggests that substrate stabilisation may be a common theme for the stabilisation of several P-450s regardless of whether or not they can be phosphorylated in the C-helix (Yu & Gunsalus, 1974; Takemani *et al.*, 1975; Kellis & Vichery, 1987). It was also seen from studies on the phosphorylation of several P-450 isoforms that nearly all the Family 2 members could be phosphorylated by PK-A; by contrast in studies of the degradation patterns of P-450 proteins it was seen that only a few possessed a biphasic degradation pattern as seen to be the case in CYP2E1 and CYP2A1 (Song *et al.*, 1989; Parkinson *et al.*, 1983). Although both rat CYP2B1 and CYP2B2 proteins were

phosphorylated both *in vivo* and *in vitro* (Koch and Waxman, 1989) these proteins did not not possess a biphasic degradation profile and were seen only to be turned over by the system acting to generate a long half life P-450 population (Parkinson *et al.*, 1983). What is not clear is how a similar phosphorylation event upon arguably similar protein structures can produce accelerated degradation of one P-450 isoform by a rapid proteolytic route, but leave another isoform still resistant to such an attack.

Section 4.9.7: Other systems to assess a role for serine 129

The results obtained from this experimental system demonstrated clearly that the presence or absence of serine 129 produced no effect on the accumulation or time of retention of the Cyp2e1 protein. Although not affecting the lack of mutant Cyp2e1 accumulation in the culture system, the precipitous loss of the Cyp2e1 protein at day six, rather than a steady decay of protein levels as might be expected following loss of the vector, suggests that events in the cells associated with continuous culturing following transfection may effect the Cyp2e1 protein degradation. Although the COS 7 cells were seen to be approximately as viable at day six as at day seven it is possible that, on having reached confluency around this time, the cells may activate new degradative systems potentially associated with ageing; age-related down-regulation of P-450 has been noted *in vivo* in rats for example (Marle *et al.*, 1990). Alterations in the levels of activity of the autophagasomal pathways have been associated with starvation for example (Dice *et al.*, 1986; Dice *et al.*, 1987; Mortimore *et al.*, 1988) and are also seen to occur in similar circumstances in other eucaryotic systems such as in *S. cerevisiae* (Hirsch *et al.*, 1989). To assess this possibility and so obtain clearer data concerning the time span of the mutant and native Cyp2e1 proteins, it would be necessary to generate a system which removed the requirement for long-term cell culture. One possibility would be to use inducible expression vectors to generate the Cyp2e1 expression; in this manner a pulse of Cyp2e1 protein could be produced over a much shorter period and its loss examined without the need to wait initially for the loss of the transiently expressing plasmid construct with a constitutive promoter activity. Further refinements to this system may be to generate a more unstable heterologous message, for example by introducing destabilising UAAAU elements into the 3' UTR (Section 4.5.1) and so reduce further the time period over which a pulse of Cyp2e1 protein would be produced.

The results from this experiment suggest that serine 129 plays no role in the stability of the Cyp2e1 protein in the study system employed; it is not clear however whether this is also the case for the substrate-induced stabilisation of the protein observed *in vivo*, as

the results obtained could be argued to reflect the extinction of the rapid proteolytic degradative system in the tissue culture cell line.

Section 4.10: The induction of CYP2E1 by hyperbaric oxygen: generation of CYP2E subfamily protein stabilisation without a substrate

As well as acting as monoxygenases, P-450s can also act as oxidases through the reduction of oxygen to hydrogen peroxide or water; this observation suggested a role for P-450 in the generation of oxidative stress by the production of hydrogen peroxide and the generation of free-radical damage to membranes and other cellular components (Gorsky *et al.*, 1984). It was noted that exposure of rats to 95% oxygen led to an increase in the concentration of P-450 in both the lung and liver by around 50%. Further analysis of the specific isoforms of P-450 associated with this phenomenon illustrated that 60% of the total induction could be attributed spectrally to an induction of CYP2E1 protein. This observation was confirmed by Western blotting; it was seen that the CYP2E1 mRNA levels were however unchanged (Tindberg & Ingelman-Sundberg, 1989).

Thus both the presence of the substrate and oxygen alone has been associated with an increase in the stability of CYP2E1 protein. It is possible that in both cases a common structural modification of the CYP2E1 protein occurs as a result of the presence of oxygen or a substrate leading to its stabilisation. In both instances the structural modification may be producing the same external signal, or lack of it, to a rapid proteolytic system.

Other observations however may cloud this hypothesis of a common inductive pathway for oxygen and CYP2E subfamily substrates by means of altered CYP2E protein stability. It was seen in the case of super-oxide dismutase induction by hyperbaric oxygen that the elevations in protein which were seen resulted from an increase in message translatability (Hass *et al.*, 1987). In this context, the possible control of CYP2E translatability, as suggested to occur on exposure to solvents (Kim *et al.*, 1990; Kim & Novak, 1990), may have a role in the elevation of CYP2E1 protein seen. It is an intriguing possibility that factors may interact with the conserved structure and potential $NF_{\kappa\beta}$ recognition site within the 5' UTR of the CYP2E subfamily in these conditions (Section 5.4.4, Figures 5.7a and 5.7b). It has been shown that the activity of the $NF_{\kappa\beta}$ transcription factor itself is tightly controlled by levels of oxidative stress and a similar activity may be displayed by other factors capable of binding $NF_{\kappa\beta}$ recognition sites (Meyer *et al.*, 1993).

Section 4.11: Transcriptional control of the CYP2E subfamily genes

In both the starved mouse (Section 4.5) and diabetic rat (Section 3.4.1) the levels of CYP2E subfamily transcripts have been seen to be elevated; in the chemically-induced diabetic rat the transcript elevation has been suggested to relate to a stabilisational event (Song *et al.*, 1989). Arguments have been presented against this suggestion based on the nature of the control transcript used in these experiments (Section 3.7.3) and on the observed retention of mRNA induction in the starved mouse even though the Cyp2e1 3' UTR is interrupted by a β2 SINE (Section 5.4.1) suggesting that stabilisation would not proceed by previously identified mechanisms. It remains a possibility therefore that the increases in CYP2E subfamily mRNA levels seen in these situations is mediated by transcriptional activation of the gene. Physiological signalling pathways may therefore be responsible for the activation of the CYP2E subfamily genes in response to biochemical changes detected in these physiological and pathological states. Precedents for the transcriptional regulation of the CYP2E subfamily have been seen both during early development and in the adult. Through the use of methylation sensitive *Hha* I and *Hpa* II restriction endonucleases, it has been shown in both human (Jones *et al.*, 1992) and rat development (Umeno *et al.*, 1987) that CYP2E1 gene becomes demethylated and transcriptionally activated after birth; although the demethylation of genes is often associated with transcriptional activation it is likely that it occurs as the result, rather than the cause, of transcriptional activity in the gene generated by another effector (Bird, 1984). Studies in the rabbit suggested that the two CYP2E genes were developmentally regulated in a differential manner, with CYP2E2 levels rising immediately after birth but CYP2E1 levels only appearing at week four *post partum* (Bonfils *et al.*, 1990). This is consistent with the observation of two peaks of aniline hydroxylase activity, associated with CYP2E, being seen in the rabbit, with one peak occurring immediately after birth and the second some four weeks later (Tredger *et al.*, 1976). Using the 800 base-pair partial Cyp2e1 cDNA, no Cyp2e1 transcripts could be detected in total RNA prepared from mouse embryos (Kind gift Dr R. Hill, MRC Human Genetics Unit, Edinburgh) suggesting that in a similar manner the mouse Cyp2e1 gene is transcriptionally inactive prior to birth.

It has been noted that both the mouse Cyp2e1 gene, through the indirect action of androgens in the female kidney (Hong *et al.*, 1989), and rat CYP2E1 gene, through the removal of growth hormone (Williams & Simonet, 1988; Yamazoe *et al.*, 1989a), were seen to be transcriptionally activated in the adult animal in certain circumstances.

These observations imply a clear role for transcriptional regulation in the developmental activation of CYP2E subfamily and also in adult animals in certain circumstances and

set a precedent for the possible involvement of control of the CYP2E subfamily genes in situations in which their transcript levels are seen to be elevated.

In general terms transcriptional activation mechanisms operate via either the binding *de novo* of transcription factors to controlling portions of the gene, or the modification of prebound transcription factors, leading to initiation, or acceleration, of transcription from the promoter. The controlling regions of a gene are generally seen to be located 5' of the transcriptional initiation site but this is not necessarily always the case. Although earlier studies suggested the existence of tissue specific DNA elements within a gene that were active only in the presence of tissue specific transcription factors (Maniatis *et al.*, 1987), it is now a general observation that a single DNA element within a gene can be bound by a variety of transcription factors. The factors which are able to bind this site are dependent on the tissue type, and it is the interaction between these protein elements, rather than an exclusive nature of the DNA element itself, that allows a high degree of complexity in the tissue specific expression of a gene to be established (Lenardo & Baltimore, 1989).

The possible transcriptional regulation of the Cyp2e1 gene was investigated; such a study may possibly give an insight into the similar situations in which other CYP2E subfamily genes may also be activated, and could relate to the observed developmental regulation of the gene and the processes surrounding the extinction of the CYP2E subfamily genes in tissue culture systems.

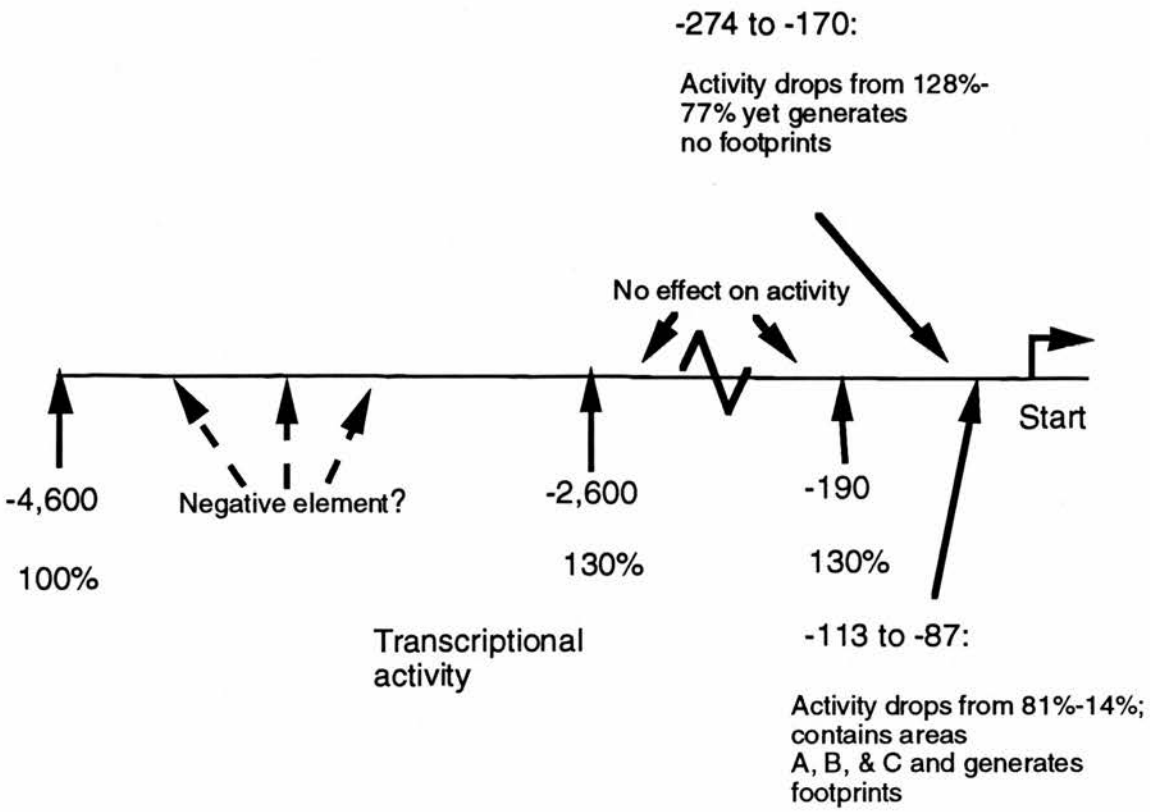
Section 4.12: Levels of homology in the 5' region of the CYP2E subfamily genes and regions important in their control

The 5' region of the mouse Cyp2e1 gene was cloned and sequenced and its level of homology to the rat CYP2E1 gene analysed (Section 5.8.5). The levels of homology were seen to be very high, sround 80%, suggesting that any controlling mechanisms operating on the genes would be conserved between species.

A study of the 5' region of the rat CYP2E1 gene has delineated potential areas of importance in the control of transcription of the CYP2E gene subfamily (Umeno & Gonzalez, 1990); the results from this study are summarised in Figure 4.14. Deletion analysis of the 5' portion of the rat CYP2E1 gene in association with an *in vitro* transcription assay demonstrated the existence of several regions important in the transcriptional activity of the gene. The *in vitro* transcription assay system involved the use of liver nuclear extracts to produce transcripts from constructs containing portions of the CYP2E1 5' region, and the transcript levels produced were quantified through the use of a primer extension assay using radioactively labelled oligonucleotide probes to the CYP2E1 mRNA generated. Using an initial construct of -4,600 to +190 base-

pairs of the rat CYP2E1 gene the basal activity, termed therefore 100%, of the gene was assessed. Deletions were then performed from the 5' most end of the CYP2E1 gene; by deleting 2 kilobase-pairs of DNA the transcriptional activity of the gene was seen to rise to 130% of the basal value suggesting the presence of a negative element between -4,600 and -2,600 base-pairs of the CYP2E1 gene. Deletion of the portion of the gene between -2,600 to -190 however had little effect on the transcriptional activity suggesting this region played little role in the control of transcription. Further deletions however revealed the presence of 2 regions which had a positive action on CYP2E1 transcription and loss of which led to a fall in transcriptional activity. These regions were -274 to -170, leading to a fall in activity from 128% to 77%, and -113 to -87, which led to a fall in activity from 81% to 14%. DNAse I protection assays of these two regions showed that no protein in the extract appeared to bind between -274 to -170, but footprints were seen in the -113 to -87 area suggesting that this region was bound by protective protein factors. The -113 to -87 portion of the promoter was seen to possess three protected areas and these were further analysed by the use of bandshift assays on oligonucleotides made to these three regions. All three areas (-120 to -93, denoted area A, -89 to -81, denoted area B, and -71 to -55, denoted area C) were seen to specifically interact with, and be band-shifted by, protein components in the nuclear extracts employed. The bandshifts generated by area A could be competed through the addition of an oligonucleotide previously known to interact with the transcription factor HNF-1 (also termed HP-1, LF-B1, and APF. Baumheuter *et al.*, 1988; Frain *et al.*, 1989). In a similar manner the band shift produced by area C could be competed through the addition of an oligonucleotide containing the site recognised by the NF-Y factor (Raymondjean *et al.*, 1988). The B region oligonucleotide however was not competed by addition of any of the oligonucleotides used in this study and was therefore suggested to represent a region bound by a factor more specific to CYP2E1. Through the use of these oligonucleotides in conjunction with the CYP2E1 transcriptional assay system it was seen that only region A led to a decrease in activity of the CYP2E1 promoter suggesting either that the factors binding regions B and C are in vast excess or are not important in the control of CYP2E1 transcription (Umeno & Gonzalez, 1990). The results from these studies are represented in Figure 4.14.

Figure 4.14: The regions of the rat CYP2E1 gene seen to be important in the control of transcriptional activity; the diagram is not to scale. **Start:** the transcript start site.



The rat CYP2E1 gene is seen to become demethylated and transcriptionally active one day after birth (Umeno *et al.*, 1987); however by using nuclear extracts from fetal, day 3 and adult rat livers it was seen that the bandshifts produced by the adult and foetal extracts were identical. When region A, the HNF-1 site, was used in the foetal and adult bandshifts it was seen however that a higher mobility band was produced by the fetal extracts than the adult. These results were taken to suggested that HNF-1 may become post-translationally modified, possibly by glycosylation, at birth and activated (Umeno & Gonzalez, 1990).

Section 4.13: Transcription factors possibly interacting with the 5' region of the mouse Cyp2e1 gene

The transcription factors which may potentially interact with the 5' region of the mouse Cyp2e1 gene were analysed. The 5' sequence of the mouse Cyp2e1 gene was analysed to see if it contained known transcription factor recognition sequences using the homology consensus searching computer program MacPattern and by using permutational changes of published transcription factor recognition sequences (Locker

& Buzard, 1990; Faist & Mayer, 1992) with the Best and Gap programs of the GCG package (Devereux *et al.*, 1984) (Figure 4.15).

These searches identified the HNF-1 (around -130) and NF-Y (around -100) sites suggested to exist by bandshifting studies of the rat CYP2E1 gene (Umeno & Gonzalez, 1990), as well as the presence of several other sites within the Cyp2e1 gene potentially capable of affecting transcriptional activity (Figure 4.15). The Cyp2e1 gene is predicted to possess a TATA box (around -60) suggested by the presence of several TATA box and viral proteins recognition sites around this area; the TATA box binds the TFIID protein and directs the positioning of the RNA polymerase II (Nickolov *et al.*, 1992; Greenblat, 1992). Upstream from this region, the Cyp2e1 gene is C-rich suggesting that factors like Sp1, which are often associated with the TATA box, may also bind this region (Briggs *et al.*, 1986). Upstream (around -100) from the potential TATA box the Cyp2e1 gene is suggested to bind CCAAT motif recognising proteins such as NF-1A and NF1-B as well as the NF-Y factor. Upstream of the proposed HNF-1 site (around -140) is a predicted GATA recognition site (around -160); these elements are often associated with the development of tissue-specific gene expression (Ho *et al.*, 1991; Ito *et al.*, 1993).

Alignment of the rat CYP2E1 and mouse Cyp2e1 promoter sequences shows that the region termed B, suggested to interact with a more specific CYP2E1 transcription factor in the transcription assay study of the rat gene (Umeno & Gonzalez, 1990), is lost in the mouse gene; this region forms the only gapped site in this portion of the otherwise highly conserved sequences (Figure 4.15). This suggests that either this factor does not play a significant role in the regulation of rat CYP2E1, as indicated by oligonucleotide competition studies (Umeno & Gonzalez, 1990), or that this portion of the rat gene generates a possible divergence in the control of the gene in the two species. It has previously been noted that such differences may occur, for example the rat gene is apparently transcriptionally activated by a fall in the level of growth hormone (Yamazoe *et al.*, 1989a) whereas transcription from the mouse gene is unaffected (Hong *et al.*, 1990).

Although in the rat CYP2E1 gene, removal of the -2,600 to -190 portion of the 5' region was not seen to affect the basal promoter activity (Umeno & Gonzalez, 1990) there are potential recognition sites within the portion of the gene highlighted by the homology-based computer analysis. A putative AP2 site, flanked by cAMP responsive element binding sites, was seen around -320 in the Cyp2e1 promoter; AP2 binding factors have previously been shown to interact with cAMP responsive elements (Imagawa *et al.*, 1987).This portion of the gene is highly conserved between the rat and the mouse with only one base change within the AP2 site binding consensus at this site.

All the CYP2E subfamily genes contain a near consensus NFκβ recognition sequence just upstream of the initiation codon. This recognition sequence provides a good example of the observation that a single site may be bound by a variety of protein factors dependent on those present in a specific tissue. The NFκβ recognition sequence has previously been shown to be bound by a series of factors including the *rel* family (Levin *et al.*, 1991) of proteins (NFκβ, *c-rel* , *v-rel* , and *dorsal*), as well as a series of unrelated factors such as C/EBP (Clark *et al.*, 1988), MBP-1 (Baldwin *et al.*, 1990), KBF-1 (Israel *et al.*, 1987), and H2TF1 (Baldwin *et al.*, 1987) and has been discussed previously (Section 5.4.4). The predicted transcriptional recognition sites are illustrated in connection with the aligned mouse Cyp2e1 and rat CYP2E1 5' region sequences in Figure 4.15.

Section 4.13.1: The nature of the transcription factors suggested to interact with the 5' region of the Cyp2e1 gene

The nature of the putative transcription factors suggested to interact with the mouse Cyp2e1 gene by computer analysis and the common factors suggested to interact with the rat CYP2E1 gene by transcriptional assay (Umeno & Gonzalez. 1990) may give an insight into the potential physiological, developmental and extinction-related controls operating on this subfamily. Several of the recognition sites and the variety of proposed factors which may bind to them are reviewed and the relevance of the sites and possible binding factors to these aspects of the CYP2E gene subfamily are discussed.

HNF-1 recognition site (consensus: GTTAATNATTAAC): The HNF-1 transcription factor has been shown to interact with a variety of liver specific genes including albumen, C-reactive protein, aldolase B, pyruvate kinase, α-fetoprotein, α- and β-fibrinogen, and α_1-antitrypsin (Cereghini *et al.*, 1990). HNF-1 is a member of the homeobox family of proteins and as such contains the helix-turn-helix homeobox motif commonly seen in proteins associated with developmental regulation (Dressler & Gruss, 1988). Its role has been characterised in connection with the control of the albumin gene in the liver; the albumin gene, in common with CYP2E subfamily genes, contains predicted binding sites for NF-Y and HNF-1, as well as C/EBP (Cereghini *et al.*, 1990). In a manner similar to that observed for CYP2E subfamily and in common with a variety of P-450 genes the albumin gene is seen to become "extinct", i.e. it is no longer transcriptionally active, in certain stable liver cell lines and notably in poorly differentiated or de-differentiated liver cell lines. In de-differentiated liver cells, although the albumen gene was transcriptionally inactive, a factor was still seen to bind to the HNF-1 recognition site; this factor, termed vHNF-1 (variant), was shown to

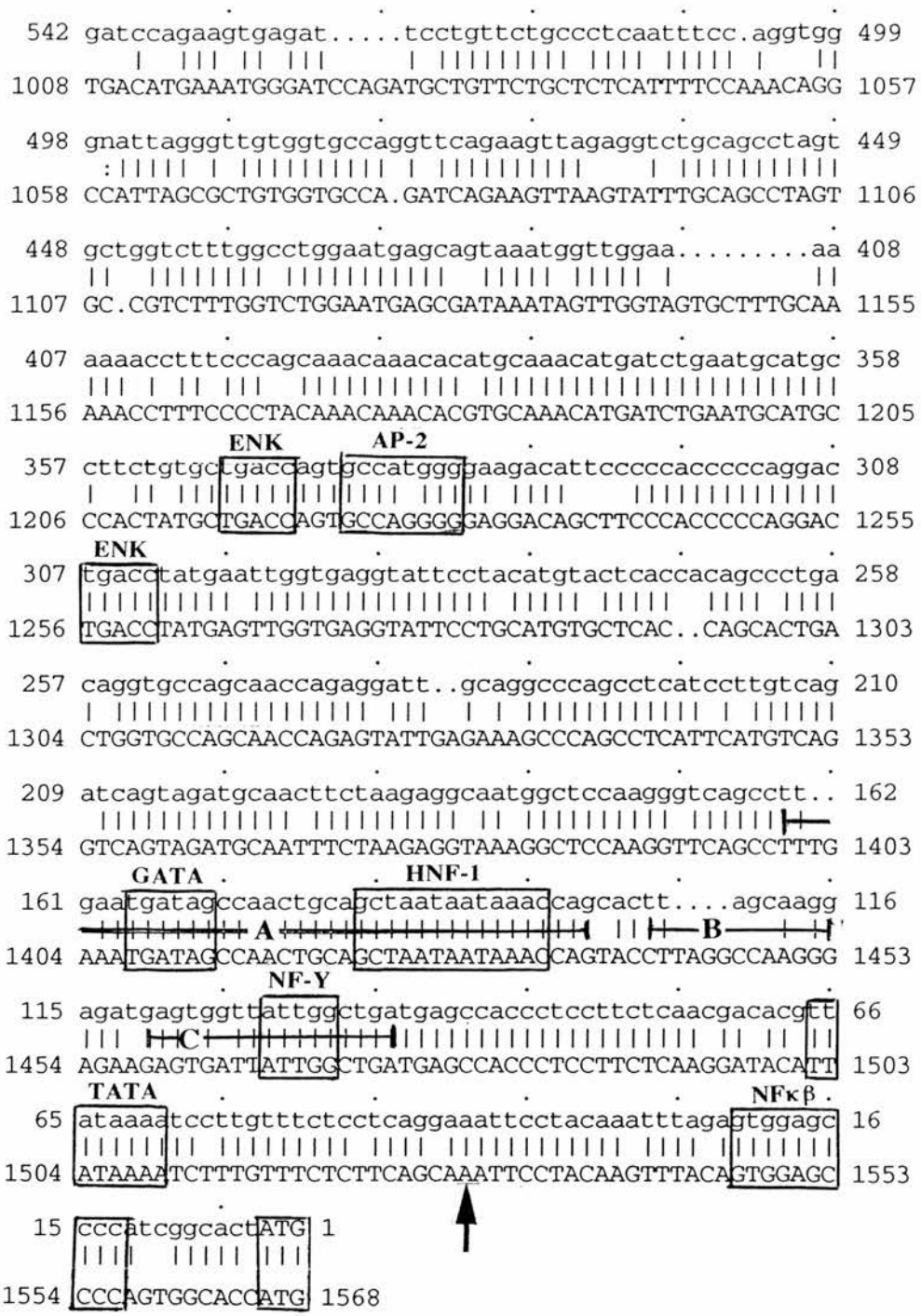


Figure 4.15: A comparison of the 5 ' region of the mouse Cyp2e1 (upper) and rat CYP2E1 (lower) promoter regions. The sequences were aligned using the GCG Gap alignment program and the gapped similarity between the two sequences is 85.53%. The areas of the mouse sequence, and the corresponding rat sequence, predicted to potentially encode transcription factor binding sites are boxed. The translation initiating methionine codon of the two CYP2E subfamily transcripts is boxed and the transcriptional initiation site of the rat CYP2E1 gene is arrowed. The three areas of the rat gene, **A**, **B**, and **C** investigated by bandshift assays (Ueno & Gonzalez, 1990) are underlined.The nature of the transcription factors potentially relevant to the control of the CYP2E subfamily genes, their consensus binding sites, and the nature of the A, B and C sequences are detailed in the text.

bind the same site as HNF-1 but generate a higher mobility band in a bandshift assay. cDNA clones for both HNF-1 and vHNF-1 have been obtained showing them to be two distinct proteins, which nonetheless possess identical DNA recognition sites, and both proteins were shown to be present in the adult liver (Bach *et al.*, 1991). This observation suggests that the higher mobility band produced on the HNF-1 recognition site in the rat CYP2E1 gene by foetal rat liver extracts (Umeno & Gonzalez, 1990) may relate to a developmental switch between vHNF-1 and HNF-1 in the initiation of transcription from the CYP2E1 gene *post-partum* and not, as suggested, to a post-translational modification of HNF-1 itself. Examples of pairs of transcription factors with antagonistic actions similar to that observed for HNF-1/vHNF-1, are seen in the development of tissue-specific gene expression. The transcription factors Oct-1, the effective equivalent of the inactivating vHNF-1, and Oct-2, the effective equivalent of the activating HNF-1, have been seen to control the development of certain B-lymphocyte specific genes (Muller *et al.*, 1988; Scholer *et al.*, 1989).
It was seen that the more differentiated a cell line was, the higher the HNF-1: vHNF-1 ratio present; well differentiated rat liver cell lines cells such as HepG2 expressed both proteins whereas poorly differentiated cells, such as the H5 line, expressed only vHNF-1 (Cerighini *et al.*, 1990). This observation emphasises the fact that the same DNA site can be bound by different transcription factors producing entirely different transcriptional results. This observation also suggests that the possible reason for the extinction of P-450 and other genes may due to transcription factor de-differentiation and loss in the cell lines; other transcription factors have be seen to be lost in cell lines, such as C/EBP (Friedman *et al.*, 1989).

AP-2 recognition site (Consensus: $CCC^{A}/CN^{G}/_{C}{}^{G}/_{C}{}^{G}/_{C}$): An AP-2 recognition site was observed in a variety of genes including metallothionein IIA, collagenase, *c-myc* , growth hormone and pro-enkephalin and these recognition sites have been implicated both in the developmental and adult control of gene expression (Winning *et al.*, 1991). The recognition site is bound by the AP-2 factor itself, as well as a variety of other factors such as NF-1 (Cantois *et al.*, 1990). An AP-2 recognition site has been seen to be present in the cAMP responsive regions of many genes and all the genes it has been noted in previously have been responsive to either phorbol ester or cAMP modifications (Imagawa *et al.*, 1987). In the human pro-enkephalin gene for example an AP-2 site was seen 10 base-pairs downstream of two cAMP responsive elements and around 100 base-pairs upstream from the transcription start site; removal of the AP-2 site reduced the cAMP inducibility of the gene suggesting that the factor binding the AP-2 site interacts with the cAMP responsive factors to generate the response but may not itself be directly responsible for the induction (Hyman *et al.*, 1989). The cAMP responsive

element motifs associated with the AP-2 site in the pro-encaphalin gene (CGTCA) were used in a search of the Cyp2e1 gene; this revealed the possibility of two such elements just proximal and distal to the potential AP-2 site and conserved in both the Cyp2e1 and the rat CYP2E1 5' regions and annotated as **ENK.** in Figure 4.15.
The ability of AP-2 factor to interact with DNA has seen to be reduced by CpG methylation (Coub & Goodman, 1990); both the rat (Umeno *et al.*, 1988b) and human (Jones *et al.*, 1992) CYP2E1 genes have been shown to be methylated before birth and this methylation may therefore be interfering with the ability of factors, potentially like the AP-2 factor, to bind the promoter. The AP-2 factor is seen to interact with other nuclear proteins such as the SV40 large T antigen,which prevent its interaction with DNA (Mitchel *et al.*, 1987); this binding of factors important in the generation of P-450 transcription by other transcription factors, either endogenously expressed as a result of de-differentiation or related to the method of establishing the cell line, may play a part in their extinction in tissue culture systems.

GATA recognition site (consensus: $^{T}/_{A}GATA^{A}/_{G}$): The GATA recognition sites are bound by a family of transcription factors important in the development of cell specific gene expression, and have been associated with the generation of tissue specific gene transcription in lymphocytes (Ho *et al.*, 1991), endothelial cells (Wilson *et al.*, 1990), testes (Ito *et al.*, 1993) and the brain (Yammamoto *et al.*, 1990). The potential presence of such a site in the Cyp2e1 gene may relate to the widespread expression pattern of the Cyp2e1 protein (Figure 4.3); expression of CYP2E1 protein in other species has also be seen in diverse tissues such as the rabbit bone marrow (Schnier *et al.*, 1989) and the rat brain (Hansson *et al.*, 1990).

NF-Y recognition site (consensus $CTN_6{}^{A}/_{G}{}^{A}/_{G}CCAATCAN^{C}/_{T}{}^{G}/_{T}$): NF-Y, or CP-2, recognition sites are seen as a common component of both generally and tissue specifically expressed genes. A variety of transcription factors recognise this site and many factors, such as TF-1 or C/EBP, have been shown to bind it although only the NF-Y protein itself to date was shown to have an absolute requirement for the NF-Y site. An NF-Y factor cDNA has been isolated showing it to be highly homologous to the yeast HAP transcription factor suggesting that these proteins play a highly conserved general role in eucaryotic transcription (Hooft van Huijsduijen *et al.*, 1990). Factors binding to the TF-Y recognition site are unlikely to be the actual point at which a gene may be regulated, but factors important in transcriptional regulation may exert their action via NF-Y site factors. An example of this is seen in the human heat shock gene hsc70 promoter which is regulated by the E1a transcription factor mediating its actions through the CCAAT binding factor (Agoff *et al.*, 1993).

$NF_{\kappa\beta}$ recognition site (consensus $GGGA^{A}/_{C}TN^{T}/_{C}CC$): The $NF_{\kappa\beta}$ recognition site provides one of the clearest examples of a single DNA region being bound by a series of different transcription factors. The site is bound by members of the *rel* gene family, which includes $NF_{\kappa\beta}$, *v-* and *c- rel* , and *dorsal* , as well as a variety of factors unrelated to the *rel* family. $NF_{\kappa\beta}$ itself is the best understood factor which binds this element and is associated with the control of several classes of genes in the immune system, acute phase and inflammatory processes although it has also been detected in a wide series of non-immune related cells. The $NF_{\kappa\beta}$ factor is composed of a heterotetramer comprised of two DNA binding and two non-DNA binding proteins; prior to activation this heterotetramer is held in an inactive complex in the cytoplasm by a group of binding proteins. A variety of signals, including mitogens, cytokines, oxidative stress and interleukins in the immune system and tumour necrosis factor and phorbol esters in non-immune system cells, produce phosphorylation changes in this latent complex leading to release of $NF_{\kappa\beta}$, nuclear localisation and DNA binding (Meyer *et al.*, 1993). The *Drosophila* development controlling factor *dorsal* is controlled in a similar manner by its retaining factor termed *cactus* ; similar mechanisms of control have been seen for the *c-* and *v-rel* proteins (Lewin *et al.*, 1991).

Non-*rel* related factors which will bind to $NF_{\kappa\beta}$ sites include H2TF-1 (Baldwin *et al.*, 1987) and EBP-1 (Clark *et al.*, 1991), both associated with the MHC enhancer in the HeLa cell line, and KBF-1 (Israel *et al.*, 1987), found in thyroid cell extracts. MBP-1 a large protein recognising the $NF_{\kappa\beta}$ was seen in HeLa, fibroblast, retinal and the HepG2 liver cell lines. A partial cDNA clone showed that MBP-1 contained a consensus Cys_2. X_{12}. His_2 zinc finger motif; MBP-1 protein levels were seen to be transcriptionally controlled and the message level was seen to be inducible by phosphorylation (Baldwin *et al.*, 1990). Zinc finger binding proteins are thought to be very ancient and have been shown to be capable of binding both RNA and DNA recognition sites (Dressler & Gruss, 1988).

$NF_{\kappa\beta}$ recognition sites can effect transcription even if present downstream of the transcript initiation site as has been seen in the case of the mouse *c-myc* gene where the site is present within exon 1, 440 base-pairs downstream of the start site (Kessler, *et al.*, 1992).

The potential $NF_{\kappa\beta}$ site in the CYP2E gene subfamily is both highly conserved and close to consensus and, if bound by a zinc finger recognition protein, could either be recognised within the mRNA or the DNA. The structure which contains the putative $NF_{\kappa\beta}$ site is has been discussed previously and is shown in Figure 5.7. The putative $NF_{\kappa\beta}$ site may function either in the control of the CYP2E subfamily gene transcription or possibly effect the translatability of the CYP2E subfamily mRNA.

Section 4.13.2: Implications of the factors which may interact with the Cyp2e1 gene 5' region

The computer-generated prediction of potential control elements in the Cyp2e1 promoter suggests the presence of basic control elements such as a TATA and CCAAT boxes, tissue specific regulatory sites such as the GATA and HNF-1 recognition sites, and regulatable elements such as the AP-2, its associated cAMP elements, and the $NF_{\kappa\beta}$ recognition site. The substantial overall homology between the rat and the mouse CYP2E subfamily 5' regions and the high level of conservation seen in the predicted basal, inducible and tissue specific recognition sites suggests that both the basal and regulatable control of the genes would be similar (figure 4.15). The only divergence in the promoter region between the two genes is the presence of an element, termed region B, at around -120 base-pairs in the rat gene absent in the mouse. This region was however suggested to be unimportant in a transcription assay system (Umeno & Gonzalez, 1990).

The basal elements of the Cyp2e1 gene are not unusual and may form the targets for the mediation of the control effects of other proteins. The close proximity of the tissue specific GATA and HNF-1 predicted sites suggests that possibly these elements may play an overlapping or functionally redundant role in allowing the expression of the gene in the variety of tissue types noted (Section 4.4). The predicted presence of regulatable elements suggests that signalling systems may exert an effect on the transcriptional activity of the Cyp2e1 gene. These sites may be responsive in the pathological and physiological situations where the CYP2E subfamily transcript levels are elevated and may potentially mediate their actions at both transcriptional and post-transcriptional levels.

Clearly, with the exception of the HNF-1 and TF-Y sites which have been examined experimentally (Umeno & Gonzalez, 1990), the predicted recognition sites in the Cype1 promoter are speculative and such sequence elements may have arisen by chance (Mikkelsen, 1993) or may play no function in the regulation of the gene. However the fact that these sites are conserved accross species may strengthen the argument for their possible relevance but this can only be substantiated by further experimental analysis.

Section 4.14: Cyp2e1 5' promoter assays; an investigation into a role for insulin in transcriptional control of the the CYP2E subfamily genes

Cyp2e1 mRNA levels rise in starvation (Section 4.5.2); rat CYP2E1 mRNA levels rise in diabetes which could be regarded as an extreme form of starvation (Section 3.4.1). Although initial suggestions related the elevation of the rat CYP2E1 mRNA in diabetes suggested a role for a stabilisation event (Song *et al.*, 1987) arguments presented here suggest that both this event, and so by analogy the Cyp2e1 transcript level, could be mediated transcriptionally (Section 3.7.3; Section 5.4.3).

The rat CYP2E1 gene was suggested to be transcriptionally repressed by the action of growth hormone (Yamazoe *et al.*, 1989a) and the fall in growth hormone levels was suggested to be one reason to explain the CYP2E1 mRNA increase in diabetes. Cyp2e1 transcription however appears unaffected by the levels of growth hormone (Hong *et al.*, 1990; Henderson *et al.*, 1990); given the observed high level of homology between the mouse and rat genes and the conserved response of the genes to starvation, it seems unlikely that the actions of growth hormone alone would account for the elevation of rat CYP2E1 mRNA but that a common inducing factor may be shared between the CYP2E subfamily genes to produce the increases in mRNA levels. One factor which may be the common effector in the CYP2E subfamily control is insulin; insulin has been seen previously to affect the transcriptional activity of a variety of genes involved in glycolysis and gluconeogenesis whose mRNA levels have been seen to change in diabetes and starvation. Insulin was also suggested to be involved in the stabilisation of rat CYP2E1 protein in transient tissue culture systems (Elliason *et al.*, 1990; Ingelman-Sundberg *et al.*, 1991) and a possible rationale involving an antagonism between the biochemical and hormonal regulation of CYP2E subfamily has been suggested (Section 4.8). The possibility that insulin may be involved in the stabilisation of the CYP2E subfamily protein *per se* also suggests that the hormone may play a role in the control of transcription of the genes.

The demethylation and transcriptional activation of the CYP2E genes in the rat (Umeno *et al.*, 1987), human (Jones *et al.*, 1992), rabbit (Bonfils *et al.*, 1990), and potentially mouse, may also relate to hormonal changes associated with the biochemical modifications resulting in a mammal at birth. Prior to birth, mammals receive a high carbohydrate and low fat diet from their mother via the placenta. At birth, however, there is a dramatic switch to a high fat milk diet. Gluconeogenic processes are absent in foetal rat livers with well-fed mothers but these processes can be induced by starvation of the pregnant animal (Girard, 1986; Girard *et al.*, 1987). However, gluconeogenesis is only normally detected after birth and is associated with a rise in glucagon and a fall in insulin resulting from the switch *post partum* leading to a transcriptional activation

of gluconeogenesis (Lyonnet *et al.*, 1988). Bearing in mind the proposed gluconeogenic role of the CYP2E subfamily (Koop & Cassaza, 1986) it is possible that the *post partum* switch in the diet, and its associated activation of gluconeogenesis, initiates transcription from the CYP2E gene subfamily. What is not clear from this suggestion is why the rabbit CYP2E1 gene, unlike the CYP2E2 gene, is only maximally transcriptionally active 4 weeks after birth (Bonfils *et al.*, 1990) but this may reflect a redundancy of function of the CYP2E subfamily in this species following the gene duplication event.
The nature of the actions of insulin, and some of the genes which it has been seen to transcriptionally control, are discussed in relation to the assaying of potential control mechanisms of insulin on CYP2E subfamily.

Section 4.14.1: The cellular response to insulin

In common with a variety of peptide hormones the first step in the action of insulin involves an interaction with a specific receptor on the cell surface which triggers a series of changes in the activities of the cellular signalling systems; these post-receptor events are not yet fully understood. Insulin alters the phosphorylational state of many intracellular proteins however the exact identity of the second messenger(s) which mediate these changes is unknown. Insulin is seen to reduce the intracellular levels of cAMP, and so cAMP-dependent protein kinase (PK-C) activity, through the activation of membrane-associated phosphodiesterases and this event is thought to underlie a number of its actions (Degerman *et al.*, 1987). Insulin is seen to control metabolic processes associated with the use and storage of glucose and the cessation of the release and use of fatty acids as a fuel source; its actions for example lead to the dephosphorylation of glycogen synthase leading to the activation of glycogen synthesis and the dephosphorylation and activation of the mitochondrial pyruvate dehydrogenase complex in adipocytes leading to increased Acetyl Co-A production and fatty-acid storage (Thomas & Denton, 1986). The insulin response also leads to the appearance of elevated levels of glucose transporter proteins on the surface of several responsive cell types such as muscle and adipocytes, allowing an increased uptake and use of glucose; these actions may be mediated through either a slowing of endocytosis or an acceleration of exocytosis and increases in the levels of other receptors, such as transferrin, have also been noted (James *et al.*, 1988; Tarner & Lienhard, 1987). The action of insulin also stimulates protein and DNA synthesis as well as cellular division. The actions of insulin on genes controlling cellular division, such as *c-fos* , are thought to relate to the increase in cell proliferation (Thomas *et al.*, 1982; Stumpo *et al.*, 1988).

The actions of insulin are, however, not solely confined to the generation of dephosphorylation on effector proteins. Through the intricate interactions of phosphatases and kinases contributing to the insulin signalling pathway examples of proteins becoming phosphorylated are also seen. An example of this is seen in the activation of the membrane associated phosphodiesterases and the activation of glycogen synthase phosphatase, in both these cases insulin mediates its actions by decreasing levels of phosphorylation of these proteins (Czech *et al.*, 1988; Lavionne *et al.*, 1991; Dent *et al.*, 1990). The insulin receptor itself possesses an intracellular tyrosine kinase subunit which is activated on insulin binding. The potential roles of this kinase, other than mediating an autophosphorylational event, are unclear. Autophosphorylation of the insulin receptor has been suggested to lead to the generation of an active receptor which may then go on to generate another secondary signal. One suggestion for the insulin second messenger is a membrane associated glycosylated inositol phospholipid moiety which is released into the cell on activation of an insulin responsive phospholipase-C and acts, in conjunction with the associated diacylglycerol, in a similar manner to the release of phosphatidyl-inositol groups into the cell in response to a variety of other signals (Saltiel & Cuatrecasas, 1986 Suzuki *et al.*, 1991).

By whatever secondary signalling mechanisms, insulin mediates both positive and negative actions on the transcriptional activities of a variety of genes associated with glycolysis and gluconeogenesis (Pilkis *et al.*, 1988). Given the proposed endogenous gluconeogenic role for the CYP2E subfamily it is this aspect of its actions that are potentially of relevance in the transcriptional control of these genes.

Section 4.14.2: The transcriptional effects of insulin on gluconeogenic and glycolytic gene transcripts

Insulin has been shown to play a key role in the reciprocal control of glycolysis and gluconeogenesis, which it accelerates and represses respectively, and exerts some of its actions on the associated genes transcriptionally. If the CYP2E subfamily has an endogenous role in the gluconeogenic conversion of acetone (Koop & Cassaza, 1986; Section 3.10.1) it may be expected that the gene may be regulated in a similar manner to several other gluconeogenic enzymes and so potentially be repressed by the actions of insulin. Studying the mechanism by which insulin exerts this effect on these gluconeogenic genes may give an insight into a possible role for the hormone in the changes seen to occur in the levels of the CYP2E subfamily in starvation and diabetes.

The transcription rates of the three key mammalian glycolytic enzymes, glucose kinase (Sibrowski & Seitz, 1984), 6-fructose, 1 kinase (Gehnrich *et al.*, 1984), and pyruvate

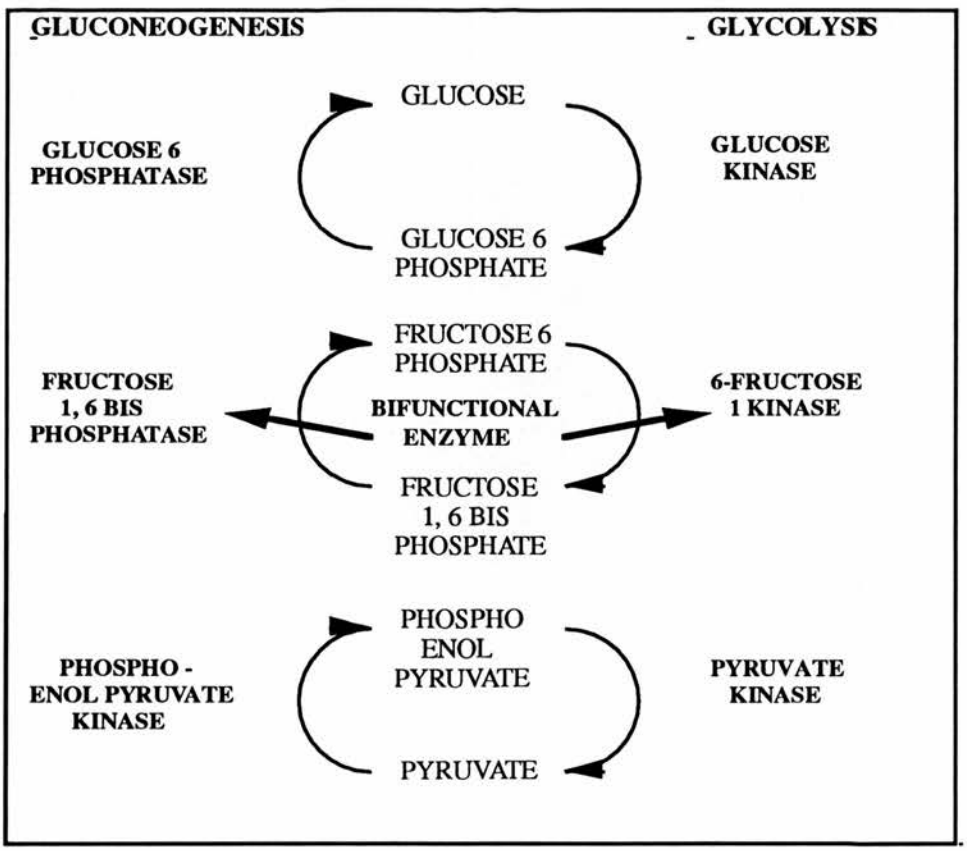
kinase (Decaux *et al.*, 1989) are seen to be elevated by the actions of insulin and repressed by elevations in cAMP levels as a result of glucagon or adrenalin/epinephrine actions. By contrast the transcript levels of the gluconeogenic enzymes phoshoenol-pyruvate carboxykinase (PEPCK) (Sasaki *et al.*, 1984) and fructose 1, 6 bisphosphatase (El-Maghrabi *et al.*, 1988) are elevated in starvation and diabetes and decreased by the addition of insulin. A message for the microsomal glucose 6 phosphatase, the third key gluconeogenic enzyme, has not yet been isolated and so the effect of insulin on the transcript levels of this enzyme are unknown. The enzymatic activity of glucose 6 phosphatase is seen to increase in starvation and diabetes (Nordlie *et al.*, 1968). The enzymatic activity of the bifunctional enzyme of the fructose 6 phosphate/ fructose 1, 6 bisphosphate cycle are seen to be increased by insulin and decrease in diabetes and starvation although the message levels are unchanged suggesting a role for regulated degradation or mRNA translatability (Colosia *et al.*, 1988; Crepin *et al.*, 1988).

Higher levels of complexity involving other hormones and metabolites are seen in the control of certain glycolytic/gluconeogenic enzymes. Pyruvate kinase, PEPCK and the bifunctional enzyme are effected by glucocorticoids, so called for their ability to promote gluconeogenesis; the levels of other components are however unchanged by their actions (Sasaki *et al.*, 1984; Decaux *et al.*, 1989; Marker *et al.*, 1989). As a general theme in the control of gluconeogenic/glycolytic enzymes it is seen that the negative actions of a signalling system are dominant over any opposing activational signals (Granner & Pilkis, 1990) for example the repressive actions of insulin are dominant over glucocorticoid positive actions in the control of PEPCK transcription (Sasaki *et al.*, 1984). The ambient levels of glucose present also appear to have a permissive effect on changes in the level of the glycolytic enzymes produced by insulin although gluconeogenic enzymes do not appear to be changed by this parameter (Granner & Pilkis, 1990).

One of the enzymes studied in most detail is the gluconeogenic enzyme PEPCK and the control of this enzyme, mediated via the antagonistic effects of cAMP and insulin, is highly complex and executed on many levels. Only in the case of PEPCK has any success been obtained in the analysis of promoter elements involved in the response of gluconeogenic genes to insulin. The difficulty in analysis of the molecular basis for transcriptional control of these processes by insulin relates to the difficulty in generating repressive insulin responses, seen to occur *in vivo* , in tissue culture study systems (Granner & Pilkis, 1990). The system used, and results obtained, in the study of the gluconeogenic PEPCK enzyme are discussed; the observed changes in the levels of PEPCK mRNA in starvation and diabetes are very similar to those seen in the CYP2E

subfamily transcripts making the possibility of a similarity in control mechanisms a possibility.

Figure 4.16: The key sites for the regulation of glycolysis and gluconeogenesis discussed in connection with the effects of insulin.



Section 4.14.3: Phosphoenolpyruvate carboxykinase (PEPCK): the transcriptional repression by insulin of a gluconeogenic enzyme

Phosphoenolpyruvate carboxykinase (PEPCK) catalyses the conversion of oxaloacetic acid to phosphoenolpyruvate, the flux generating step of gluconeogenesis (Figure 4.16). In tissue culture systems cAMP elevations and glucocorticoid actions were seen to increase the levels of rat PEPCK transcripts, through the elevation of initiation, elongation and stabilisation of pre-existing messages; glucocorticoids also increase the translation rate of PEPCK mRNA and the culmination of these changes leads to an elevation in gluconeogenesis. Insulin by contrast represses basal transcription of the PEPCK gene and dominantly reverses the inductive effects of glucocorticoids and

cAMP; the repressive actions of insulin are mimicked by phorbol esters and diacylglycerol (Kahn *et al.*, 1989).

The promoter of the rat PEPCK gene was analysed in promoter assay constructs in rat H4IIE liver cell lines leading to the mapping of regions responsive to glucocorticoids and cAMP. Insulin reproduced its dominant repressive effect on the glucocorticoid induced PEPCK transcriptional elevation and a half-maximal value of 1×10^{-11} M for insulin action was obtained in these studies which is similar to that observed *in vivo* (Magnusson *et al.*, 1987; Sasaki *et al.*, 1986). Insulin decreased basal levels of PEPCK transcription to between 14 and 22 % of control levels and produced a similar level of repression on PEPCK transcription after it had been induced, around 2.5 fold, by the synthetic glucocorticoid, dexamethasone. The prior induction of the PEPCK gene in this manner enabled the repressive action of insulin to be more easily assessed (Magnusson *et al.*, 1987; Kahn *et al.*, 1989).

The actual location of an insulin responsive element was difficult to determine using promoter assays although it appeared to be present in the first 600 base-pairs of the PEPCK gene. Attempts to further delineate the insulin responsive element by deletional analysis of the promoter were not successful and it was concluded that the repressive action of insulin may be mediated through chromatin structural modifications and so could not be reproduced in the transient promoter assay systems. To investigate this possibility -600 to +69 base-pairs of the PEPCK gene, and deletions of this construct, were fused to a thymidine kinase gene and used to produce stable cell-lines. In this manner it was hoped that the resulting chromatin arrangement around the promoter may reflect more the *in vivo* structure. These studies highlighted two insulin response elements (IREs) at -468 to -271 and -271 to +69 of the PEPCK gene. Maximal insulin repression of PEPCK promoter activity was only seen if both elements were present but the more distal element appeared to be the most powerful in isolation and was further dissected in connection with transient promoter assays (O'Brien *et al.*, 1990). These studies delineated an insulin response region (IRE) between around -411 (TGG TGT TTT GAC AAC) -396 which repressed transcription in transient assay systems and this activity could be removed by site directed changes within this element. Gel retardation analysis of this site generated a band shift using liver extracts from both control and diabetic animals suggesting that insulin may mediate its actions via post-translational modifications of a pre-bound factor (O'Brien *et al.*, 1990). The IRE area overlaps a site seen to be bound by accessory factors to the two glucocorticoid elements suggesting that post-translational changes of the putative pre-bound IRE factor may change its interaction with these elements (Imai *et al.*, 1990). The more proximal IRE area did not possess any sequence elements similar to its distal neighbour suggesting

that the actions of insulin on transcription may be mediated by changes in a variety of protein factors (O'Brien *et al.*, 1990).
The transient assay approaches used to study the effect of insulin on the PEPCK promoter activity were used in connection with the promoter analysis of the putatively gluconeogenic Cyp2e1 gene promoter.

Section 4.14.4: Investigating the effect of insulin on the promoter activity of the Cyp2e1 promoter; Cyp2e1 promoter-chloramphenicol acetyl-transferase (CAT) constructs and the HepG2 cell line

Transient promoter assay experiments were performed using the Cyp2e1 promoter fused to the Chloramphenicol acetyl-transferase (CAT) reporter gene. By analogy to the PEPCK promoter analysis it would be expected that insulin might produce a repressive effect on the level of the Cyp2e1 promoter activity if it too represented a gluconeogenic enzyme controlled in a similar fashion. In whole animal studies Cyp2e1 protein was not induced by the action of the synthetic glucocorticoid dexamethasone, or by many of the other chemicals that have previously been seen to induce other P-450 levels and so the potential repressive effect of insulin could not be more easily assessed by prior induction of the promoter by another chemical (Section 4.3). The use of prior transcriptional induction, which had greatly eased the study of the PEPCK promoter (Magnusson *et al.*, 1987), was therefore not available to the study of the Cyp2e1 gene.
The promoter region of the Cyp2e1 gene from -525 to -1 base-pairs was amplified using the polymerase chain reaction (PCR) employing oligonucleotides which also contained *Xba* I and *Sal* I restriction endonuclease sites. This portion of the Cyp2e1 gene contains the predicted basal transcriptional regions of the promoter (TATA and CCAAT boxes), the potential tissue specific regions (GATA and HNF-1 site), and the potentially regulatable elements (AP2 site and flanking regions and the NFκβ recognition site). The PCR product generated was subcloned into the plasmid pBS, sequenced confirming it to represent the expected area of the Cyp2e1 gene and then directionally subcloned into the *Xba* I and *Sal* I restriction endonuclease sites of the transient reporter plasmid pCAT-Basic (Promega). The oligonucleotides used to generate this construct are shown in Figure 4.17.
The CAT reporter system allows the analysis of DNA fragments with promoter and enhancer activity in transient transfection assays in mammalian tissue culture. The transcriptional activity of the region of the gene being assessed is reflected by the level of CAT transcripts and protein produced in the assay system. CAT is a bacterial protein which confers resistance to chloramphenicol by modifying it to mono- or di- acetylated forms, an enzymatic activity not endogenously present in the mammalian tissue culture

systems employed in the assay; the acetylated forms of chloramphenicol possess differing solubilities and so can be separated and quantified. The level of CAT enzymatic activity produced as a result of heterologous expression driven by the test DNA fragment in mammalian cells is then assayed by the level of chloramphenicol modification produced; the level of activity obtained indirectly reflects the level of the transcriptional activity of the test DNA fragment (Gorman *et al.*, 1982).

Initial transient transfections of the Cyp2e1 promoter CAT construct were carried out in both the Hepa I liver (Bernhard *et al.*, 1973) and $C_3H10T1/2$ embryo (Rezinikoff *et al.*, 1973) mouse cell lines; also included in these transfections was a control plasmid, pCAT-Control, containing an SV40 promoter region upstream of the CAT gene, and a construct containing a portion of the human CYP2D6 gene promoter (encoding bases -297 to +69, kind gift of Dr C. A. D. Smith, ICRF MPG, Edinburgh). No CAT activity was detected as a result of transient transfection of any of these constructs in either of these mouse cell lines. Transfections were then carried out in the human liver HepG2 cell line; given the high level of homology between the rat, human and mouse promoters potentially important control factors, both basal and regulatory, would be expected to be conserved between these two species and so would be expected to be operational in the mouse promoter.

The HepG2 liver cell line was derived from a hepatocarcinoma, is parenchymal in morphology and was seen to be highly differentiated expressing all but one of twenty liver antigens screened for many of which, such as albumen, insulin-like growth factor receptor and the α_2-plasmin inhibitor, were seen to be extinct in less well differentiated lines (Aden *et al.*, 1979; Zannis *et al.*, 1981; Knowles *et al.*, 1980). Studies revealed that the HepG2 cell line contained a high ratio of HNF-1:vHNF-1 transcription factors, again suggesting that it retained a differentiated state (Cerighini *et al.*, 1990). The HepG2 cell line was also been shown to retain some residual P-450 enzymatic activity which was seen to be modified by phenobarbital and 3-methyl-cholanthrene suggesting that the HepG2 cell line retains components required for P-450 enzyme induction (Dawson *et al.*, 1985). However no aniline hydroxylase, an enzymatic activity associated with the CYP2E subfamily, was seen in this cell line (Grant *et al.*, 1988; Sassa *et al.*, 1987). The Hep G2 cells have previously been seen to be responsive to insulin, for example, on treatment with insulin they were seen to demonstrate an increase in insulin receptor proteins and transcripts (Hutad *et al.*, 1989).

These observations, particularly the retention of HNF-1 with its proposed interaction with the CYP2E subfamily promoter and their ability to respond to insulin, made the HepG2 a good candidate cell line in which to study the potential control of Cyp2e1 transcription by insulin. The Cyp2e1-CAT construct, control CAT construct and

CYP2D6-CAT constructs all produced CAT responses on transfection into the HepG2 cell line.

Section 4.14.5: Insulin responsiveness of the HepG2 cell line and the effect of insulin on Cyp2e1 promoter activity

The HepG2 cell line is routinely cultured in 15% (volume: volume) foetal calf serum (FCS); in an attempt to reduce the possible effects of other serum factors present in the HepG2 culture medium other groups of HepG2 cells were permanently grown in 10% and 5% FCS. In previous experiments analysing the PEPCK promoter the rat H4IIE cells were maintained in 2.5% each of new born and fetal calf serum (Magnusson *et al.*, 1987). In other experiments using the Hep3B human line, which were generated concomitantly with HepG2 line (Aden *et al.*, 1979), the cell lines were serum-starved for 6 days prior to transfection in 10% FCS (Chou *et al.*, 1989). Attempts to transform the Cyp2e1-CAT and other constructs into similarly starved HepG2 cells were not successful and so this approach was not pursued. The insulin responsiveness of the HepG2 cells was assayed haemocytometrically via the increase in cell proliferation on two equally seeded culture plates in the presence of insulin compared to control cells, and as a function of protein synthesis/proliferation, through the assaying of the protein amounts present in two equal volumes of cells grown in the presence or absence of insulin. Insulin addition had previously been seen to elevate both these parameters in the related Hep3B cell line (Chou *et al.*, 1989; Thomas *et al.*, 1982) and both of these parameters were seen to be elevated as a result of insulin addition to the HepG2 culture medium indicating that an insulin response was occurring.

The maximal concentration employed in the study of insulin suppression of the basal and dexamethasone-induced PEPCK transcription was 1×10^{-7} M insulin (Magnusson *et al.*, 1987) and this insulin concentration was used to study the potential repressive effect of insulin on the Cyp2e1-CAT construct. HepG2 cells were transfected with Cyp2e1-CAT, pCAT-Basic (the parental vector of Cyp2e1-CAT without the Cyp2e1 5' region), pCAT control, and CYP2D6-CAT constructs in 5% or 10% FCS in the presence or absence of 1×10^{-7} M insulin. The medium containing the transfected DNA was left on the cells overnight and then replaced with medium containing the appropriate FCS and insulin concentration and the cells were left for another day to allow CAT expression before harvesting. The protein concentrations of the various samples were normalised, as the cultures containing insulin had increased cell and protein concentrations as a result of insulin's actions, and the level of CAT activity present in an equal protein aliquot of each transfection experiment was assessed (Figure 4.17).

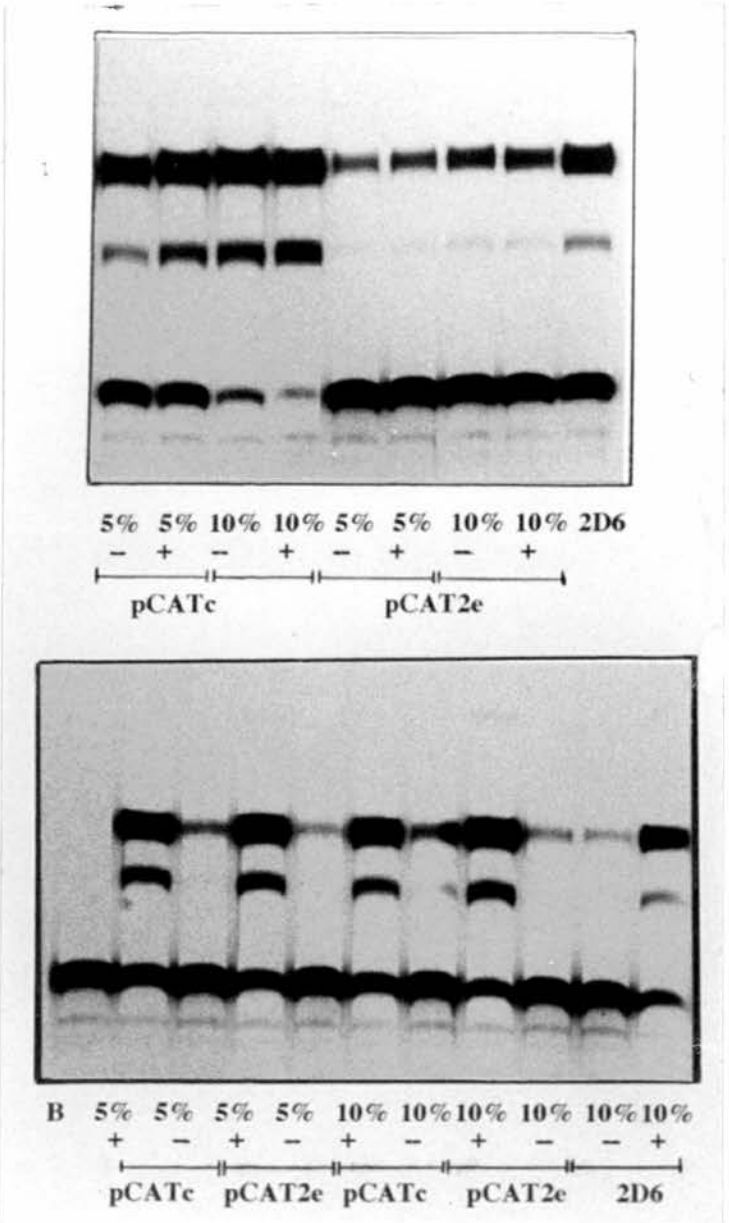


Figure 4.17: Investigating the effect of insulin addition on the transcriptional activity of the Cyp2e1 5 ' region using a chloramphenicol acetyl transferase (CAT) assay system. Hep G2 cells were transfected with: **pCATc:** control CAT vector. **pCAT2e:** pCAT basic (b) vector containing the -525 to -1 region of the Cyp2e1 gene. **2D6:** pCAtb containing the the -297 to +69 region of the human CYP2D6 gene. **B:** pCATb alone. Cells were transfected in the annotated conditions: **10% / 5%:** the amount (v/v) of fetal calf serum present. **+ / - :** in the presence or absence of 1 X 10^{-7} M insulin. Samples in the upper box were normalised for protein content befor assaying; samples in the lower box were assayed as equal volumes of cell lysate indicating the inductive effect which the presence of insulin has on the level of CAT protein present in the samples.

Oligonucleotide sequences used to generate the 5 ' region of the Cyp2e1 gene:

	Xba I	
-1 to -16 of the Cyp2e1 gene:	TCGTAGA	AGTGCCGATGGGGCTCC
	Sal I	
-532 to -516 of the Cyp2e1 gene:	GGTCGAC	AAGTGAGATTCCTGTTC

Although an insulin response was recorded in all the cultures to which insulin had been added by virtue of an increase in total cellular protein there was no unequivocal difference in the levels of transcriptional activity of the Cyp2e1 promoter in the presence or absence of insulin; these experiments were repeated in duplicate three times. The level of CAT activity produced by transient transfection, both with normalised and non-normalised protein levels to illustrate the inductive effect of insulin on both the level of cell proliferation and protein synthesis, are shown in Figure 4.17.

Section 4.14.6: The Cyp2e1 gene, a putative gluconeogenic enzyme displaying no detectable repression of basal transcription by insulin

The observed *in vivo* control operating on the Cyp2e1 transcript levels in starvation (Section 4.5) and the proposed endogenous gluconeogenic role for the CYP2E subfamily (Koop & Cassaza, 1986) had led to the suggestion that, by analogy to other gluconeogenic enzymes, insulin may repress basal transcriptional activity in this subfamily. However such an effect was not seen in the reporter system employed in conjunction with 525 base-pairs of the Cyp2e1 gene; this observation may be accounted for in part by a variety of reasons.

One possibility is that the CYP2E subfamily genes are not insulin responsive; in this instance then other common factor(s) must be responsible for the inductive patterns seen in this group of genes in starvation and diabetes. A variety of technical reasons in the assaying of insulin responsiveness in a gene means that this is by no means the only explanation.

In assaying the suppressive action of insulin on the PEPCK gene the effect was more easily seen only following prior induction of transcription with dexamethasone; this prior induction allowed an easier interpretation of the fall in transcriptional activity induced by insulin (Magnusson *et al.*, 1987). This initial augmentation of the transcription rate is not available in the study of the Cyp2e1 gene as neither dexamethasone nor a variety of other P-450 inducing chemicals produced any effect on Cyp2e1 levels (Section 4.4). As can be seen by the comparison of Cyp2e1-CAT constructs activity relative to that of the pCAT-Control a decrease in activity by the action of insulin was being sought on the background of an already fairly low transcriptional rate (Figure 4.17). The human CYP2E1 gene promoter (-539 to +8 base-pairs) was analysed in a luciferase reporter system where similarly its activity was seen to be very low with the control vector, a Rous Sarcoma virus long terminal repeat, being 100-fold more powerful in a rat hepatoma cell line (Umeno *et al.*, 1988). It is difficult to conceive of a way of augmenting the Cyp2e1 basal transcription rate given the lack of responsiveness of the gene to chemical inducers. The Cyp2e1 gene was seen

to be transcriptionally activated by the indirect action of androgens in the female mouse kidney but this effect took 8 days to occur making it an unlikely candidate for study in primary renal lines and this control is potentially unique to the kidney (Henderson *et al.*, 1990; Hong *et al.*, 1990). However attempts to mimic the effects of glucagon or adrenalin/epinephrine via the elevation of intra-cellular cAMP levels and studying their effects on the basal activity of the Cyp2e1 gene would be of interest; by continuing the gluconeogenic analogy for CYP2E subfamily, such an elevation of cAMP might be expected to induce the transcriptional activity of the gene. It is clear that this is not seen in dexamethasone administration however (Section 4.4.1)

It is possible that the HepG2 cell line retains enough of a differentiated phenotype to allow basal transcription of the Cyp2e1-CAT construct by CCAAT, TATA and HNF-1 binding factor activity but other proposed inducible factors, such as the AP2 and $NF_{\kappa\beta}$ recognition site binding factors may be lost. It may be the factors which bind these sites which are important for the mediation of the repressive actions of insulin. The lack of aniline hydroxylase activity in the HepG2 cell line (Sassa *et al.*, 1987) suggests that the endogenous CYP2E1 gene may have become extinct; such an extinction may have occurred as a result of the loss of certain protein factors needed to keep the gene active (Section 4.13.2). Studies on the PEPCK promoter in tissue culture demonstrated that the co-expression of nuclear factors associated with the regulation of the promoter increased the levels of activity obtained suggesting that the presence of key response factors may be necessary to allow an insulin response to be generated (Roesler *et al.*, 1989).

The lack of response may relate to the loss involved in the generation of the insulin signal rather than the transcription factors themselves. Cell lines such as HeLa, which were seen to contain the cAMP response factors CREB and ATF-1, still did not generate a PEPCK response on glucocorticoid stimulation and this observation was suggested to relate to a breakdown in certain signalling components normally responsible for the transduction of the insulin response (Roesler *et al.*, 1993).

Several other studies of the effect of insulin on PEPCK transcription could not reproduce its suppressive effect even in cell lines closely related to H4IIE. These observations suggest that loss of a specific factor in dedifferentiation may produce profound effects on the results obtained between cell lines (Wynshaw-Boris *et al.*, 1986). Attempts to study the inductive of insulin on transcription from the glucose-kinase promoter, which is elevated by insulin *in vivo* , proved unsuccessful *in vitro* due to the lack of a cell line that would reproduce this effect (Garner & Pilkis, 1990). These observations, in conjunction with the observed heterogeniety of the factors binding to IREs in the PEPCK gene (O'Brien *et al.*, 1990), suggest that an insulin response can be lost in a tissue culture system as a result of the loss, through

extinction, of one or more of a variety of cellular signalling components. It is only through the use of a wide range of cell lines that the lack of a Cyp2e1-CAT insulin response reflected the lack of a factor in the tissue culture system could be addressed. Alternatively the HepG2 cell line could be transfected with constructs containing the characterised PEPCK IRE to establish the intactness of an insulin to transcription factor signalling link (O'Brien *et al.*, 1990); this would however presume that the same signalling pathways would be operative on the Cyp2e1 promoter which clearly may not be the case.

The lack of Cyp2e1-CAT response to insulin may also be due to a lack of inclusion of an IRE(s) in the promoter construct and may require sequences more distal to the initiation site than those included. However to date the observed IREs have been shown to be within the size constraints of the promoter used in these studies with the repressive elements for PEPCK being seen around -411 and -278 and the inductive elements of fructose 1, 6 bisphosphatase around -169 (El-Maghrabi *et al.*, 1988). The inclusion of a larger Cyp2e1 promoter fragment may address this possibility.

The exploitation of the observation that the CYP2E genes in rabbit (Bonfils *et al.*, 1990), rat (Umeno *et al.*, 1987), human (Jones, *et al.*, 1992) and potentially mouse are not transcriptionally active prior to birth may allow the possibility *in vivo* of successfully assaying the proposed repressive action of insulin on these genes. Gluconeogenic processes are not present in normal rat liver prior to birth but are activated *post partum* , possibly as a result of a switch in diet from carbohydrates to fats (milk) and the associated fall and rise in insulin and glucagon levels respectively (Lyonett *et al.*, 1988). It was seen however that gluconeogenesis could be induced *in utero* by starving the pregnant mother (Girard, 1977; Girard *et al.*, 1986). If the CYP2E subfamily genes are controlled by similar processes that are seen to affect gluconeogenic enzymes it would be expected that starvation of the pregnant female would lead to induction of the CYP2E subfamily genes prior to birth. Such observations have been made for the embryonic chemical induction of a variety of P-450 genes including rat CYP1A1 by 3-methyl cholanthrene (Young *et al.*, 1989), and this approach may provide firmer evidence to substantiate a role for insulin in the transcriptional regulation of CYP2E subfamily although it would not directly prove it in a manner that a promoter assay system could.

Section 4.15: Summary

The observation that the mouse Cyp2e1 subfamily is seen to be regulated in a similar manner to the CYP2E subfamily in a variety of other species has several implications. The fact that acetone leads to an elevation of Cyp2e1 protein levels, in the absence of a concomitant increase in Cyp2e1 mRNA levels, suggests that a protein stabilisation event is a common theme in the regulation of this subfamily. This regulation may be mediated through the ability of certain solvents to mimic the actions of acetone, which is the proposed endogenous substrate of the CYP2E subfamily. The observation that Cyp2e1 mRNA levels are elevated in starvation questions further the proposed role of a message stabilisation event in the induction of the CYP2E subfamily in diabetes and starvation (Song *et al.*, 1987). The fact that the 3' UTR of the Cyp2e1 mRNA is seen to be ablated by insertion of a β2 element, necessitates that a stabilisation event, if it were to occur at all, were to mediated either by the residual UTR features of the Cyp2e1 mRNA or by the coding region of the transcript itself.

The molecular mechanism for the induction of the CYP2E subfamily by substrates is unclear. One proposal suggested a role for phosphorylation of serine 129 in controlling the rate of degradation of of the CYP2E subfamily proteins. Protein stabilisation was suggested to be mediated by events leading to a reduction of this serine 129 phosphorylation; events generating reduced levels of phosphorylation were suggested to include the presence of a substrate or insulin, with its associated decrease in cAMP levels. By contrast the rate of the CYP2E subfamily protein degradation was suggested to accelerated by the elevation of intracellular cAMP by agents such as glucagon and epinephrine (Ingelman-Sundberg *et al.*, 1992). This proposed control mechanism was investigated through the generation of Cyp2e1 mutants lacking the proposed controlling serine 129 site. The results obtained from the analysis of the accumulation and point of loss of Cyp2e1 proteins with or without the proposed degradation controlling serine 129 did not substantiate these suggestions. Mutant Cyp2e1 proteins, lacking a phosphorylatable residue at position 129, were not seen to accumulate, or be retained for a longer period, when expressed in mammalian tissue culture. Treating the cells expressing the Cyp2e1 proteins with insulin generated an elevation in both mutant and native protein levels suggesting that the proposed effect of insulin either relates to its global effect on the level of protein translation in general, or is mediated at a site other than serine 129. Collectively these observations suggest either that the actions of the substrate in stabilising the CYP2E subfamily are not mediated by changes in phosphorylation at serine 129, or that the components necessary to allow this control processes to operates *in vivo* are not present in the tissue culture system used.

Analysis of the promoter of the Cyp2e1 gene, in conjunction with the rat CYP2E1 gene, highlighted the possible presence of a variety of basal, tissue specific and regulatable elements which may possibly contribute to the transcriptional regulation of the CYP2E subfamily. One interesting observation was the possible presence of an $NF_{\kappa\beta}$ recognition site present in both the promoter and the transcript. This site could affect both transcription and translation of the CYP2E genes and this observation may explain some of the anomalies attached to the regulation of the CYP2E subfamily. For example, CYP2E1 protein levels are elevated in rats following exposure to hyperbaric oxygen (Tindberg & Ingelman-Sundberg, 1989) and the translation of CYP2E1 mRNA in rats increases following solvent exposure (Kim & Novak, 1990; Kim *et al.*, 1990). Factors binding to $NF_{\kappa\beta}$ recognition sites have been seen to be responsive to oxidative stress and elevate transcription from responsive genes in these situations (Meyer *et al.*, 1993) ; the possible interaction of a protein factor with the CYP2E subfamily transcripts may allow a control step in the regulation of the subfamily to be placed at translation.
The observed similarity of the regulation of the CYP2E subfamily and several gluconeogenic enzymes, in conjunction with the proposed gluconeogenic role for this subfamily (Koop & Cassaza, 1986), prompted an analysis of the potential repressive action of insulin on the transcriptional activity of the Cyp2e1 promoter. The presence of insulin however did not modify the basal level of Cyp2e1 transcription; the limitations present in this assay system, particularly relating to the inability to generate a prior induction of Cyp2e1 transcriptional activity, mean that a role for insulin in the control of transcription in the CYP2E subfamily can not however be firmly ruled out.
Collectively these observations suggest that the regulation of the CYP2E subfamily is conserved between species suggesting that situations in which the levels of the CYP2E subfamily are elevated in experimental animals, resulting in a concomitant increase in the potentially damaging metabolic activities associated this subfamily, could relate directly to the control of the subfamily in man. The details of the molecular mechanisms by which the controls of the CYP2E subfamily are executed however remain occult.

Chapter 5: Molecular Characterisation and Analysis of the mouse Cyp2e1 subfamily

Section 5.1: Introduction and Aims

To enable a study of the regulation of mouse the Cyp2e subfamily and to ascertain the level of homology, and so potentially the level of functional conservation, a mouse Cyp2e1 cDNA was cloned. cDNA sequences had previously been obtained of CYP2E1 from human, rat (Song *et al.*, 1986) and rabbit (Khani *et al.*, 1986) and CYP2E2 from rabbit (Khani *et al.*, 1987). The Cyp2e1 cDNA was used to generate heterologous expression of Cyp2e1 protein in *E. coli*, *S. typhimurium* and *S. cerevisiae* in an attempt to enable the characterisation of the potential role of the CYP2E subfamily in the generation of mutagenicity following exposure of the organism to chemicals such as nitrosamines. The Cyp2e1 cDNA was also used to clone and characterise the Cyp2e1 gene, portions of which were sequenced. The 3' portion of the Cyp2e1 gene was used in the generation of a construct to allow targeting and deletion of the Cyp2e1 gene from the genome of the mouse. Using the combination of these approaches both the level of homology in the CYP2E family and its potential role in carcinogenesis could be studied.

Section 5.2: Cloning a mouse Cyp2e1 cDNA

A full-length human CYP2E1 cDNA (Song *et al.*, 1986; Kind gift Dr F. J. Gonzalez, NCI, Bethesda) was radiolabelled and used as a probe to screen an adult male C57BL/6 mouse liver cDNA library contained within a bacteriophage λgt11 vector (Clontech, Palo Alto, CA.).The library and host cells were titrated to enable the use of a host cell: phage ratio allowing single plaques to be isolated from the library plates. Phage obtained from two plaques cross-reacting to the human CYP2E1 cDNA probe on duplicate lifts of the plated library were purified by further rounds of screening until they contained single pure clonal phage (i.e. they were made "plaque pure"). Fourteen other plaques, isolated through their cross-reactivity to the human CYP2E1 probe, were also obtained but were not processed through these further rounds of screening. Phage DNA was prepared from the two plaque pure clones and the recombinant cDNA insert released on restriction endonuclease digestion with *Eco*R I. The clones contained independent inserts of around 600 and 800 base pairs respectively. These fragments were isolated and subcloned into the *Eco*R I restriction endonuclease site of the plasmid vector pUC18 and then the replicative form of the filamentous bacteriophage-based vector M13mp18 and 19. Single-stranded DNA was prepared from the recombinant insert containing M13mp18 and 19 and sequenced. The clones did not encode a

full-length Cyp2e1 cDNA. The 600 base pair insert spanned from within exon 6 to exon 9 (base-pairs 922 to 1545 on the full-length mouse cDNA clone) and the 800 base pair insert spanned from within exon 5 to within exon 9 (base-pairs 720 to 1458 on the full-length mouse cDNA clone) by comparison with the rat CYP2E1 sequence (Song *et al.*, 1986). In an attempt to obtain a full-length Cyp2e1 cDNA clone the insert sizes in the remaining fourteen primary positives were further analysed. These clones were screened until plaque pure and then their insert sizes assessed. Using oligonucleotides which hybridise to sequences within the left and right arms of λgt11 in conjunction with the polymerase chain reaction (PCR) with an aliquot taken from the plaque pure λgt11 clone-stock as a template, the insert sizes could be quickly assessed. The results from this analysis indicated that all the inserts appear to be further copies of either the 600 or 800 base pair fragments originally isolated (Figure 5). This observation suggests that the commercially produced cDNA library has been heavily amplified and is unlikely to contain a full-length mouse Cyp2e1 cDNA.

Section 5.2.1: Generation of a BALB/c liver cDNA library in λZAPII

In order to obtain a full-length Cyp2e1 cDNA clone a cDNA library was constructed from purified poly-$(A)^+$ RNA isolated from total RNA prepared from an adult male BALB/c mouse liver. The cDNA prepared from the poly-$(A)^+$ RNA was passed through a size selecting spun-column to allow the removal of cDNA fragments of less than 400 base-pairs, thus reducing the potential background level of partial Cyp2e1 cDNA inserts. The size selected cDNA was then ligated into *Eco*R I/*Not* I adaptors: this allows the cDNA inserts to be removed from the vector using *Not*I restriction endonuclease which has a rare recognition site unlikely to occur in the cDNA itself. The size selected and *Not* I/ *Eco*R I adapted cDNAs were then cloned into pre-digested λZAP II arms and packaged. The library and host cells were titrated to enable the use of a host cell:phage ratio allowing single plaques to be isolated and the library was screened using the 800 base-pair partial Cyp2e1 cDNA as a radiolabelled probe. Two plaques which cross-reacted with the probe in a duplicate lift of the plated library were passed through further rounds of screening until plaque pure. Unlike the Cyp2e1 clones obtained in λgt11 the λZAP II clones did not require preparation of bacteriophage DNA, restriction endonuclease digestion and subsequent subcloning into a plasmid or phagemid vector. λZAP II enables the recombinant insert to be excised *in vivo* from the bacteriophage arms. The phage arms of this vector contain a divided filamentous bacteriophage f1 origin of replication with the initiation and termination functions of the origin dissected and subcloned facing each other with one functional half in each bacteriophage arm. By super-infecting the host cells containing the λZAP II

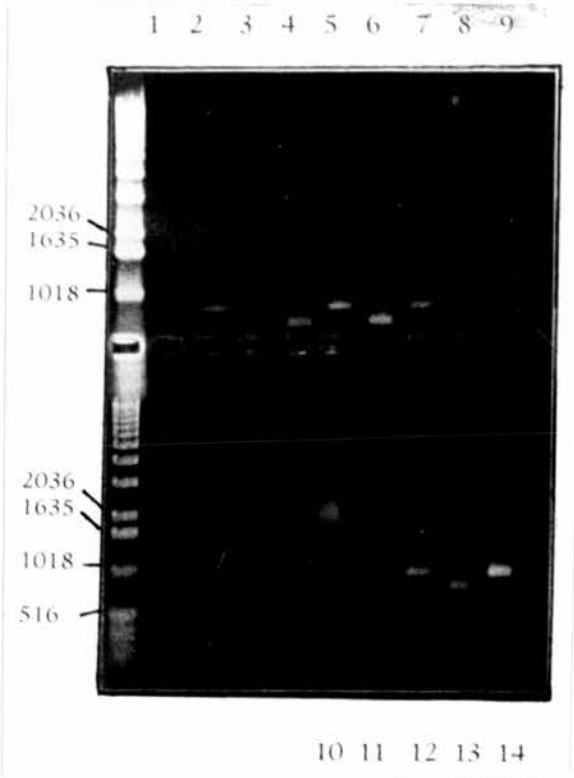


Figure 5: PCR analysis of the recombinant inserts contained within the 14 mouse cDNA λgt11 clones which hybridised with a probe generated from the human CYP2E1 cDNA. The 14 (**1** to **14**) mouse cDNA clones which hybridised with a probe generated from the human CYP2E1 cDNA were analysed for the possibility that they contained larger, or independent, inserts than those already obtained (Section 5.2). Oligonucleotides annealing to sequences within the λgt11 arms were used to amplify the recombinant insert present within the clones and the products of this reaction were analysed on an agarose gel. Marker sizes indicated are in base-pairs.

Oligonucleotides employed:

GGTGGCGACGACTCCTGGAGCCCG
TTGACACCAGACCAACTGGTAATG

clones with filamentous f1 bacteriophage, or "helper phage", proteins are produced, termed "helper proteins", which recognise the f1 sequences within the λZAP II arms and nick the DNA at the initiation sequence. DNA synthesis then begins at the nicked initiation site; as this site has been placed upstream of the recombinant insert then both the insert and another sequence, the pBS SK(-) phagemid vector, also placed downstream of the f1 initiation site are replicated before the downstream termination site is reached. The single-stranded DNA produced in this manner is circularised, leading to the regeneration of a function f1 replication origin, and packaged into phagemid particles by other proteins encoded by the super-infecting f1 phage DNA. By isolating and reinfecting other host cells with these phage particles, double-stranded pBS SK(-) plasmid containing the recombinant insert can be obtained. Preparation and *Not*I restriction endonuclease digestion of the two recombinant double-stranded Cyp2e1 clones following *in vivo* excision generated two inserts of around 1,800 base-pairs. Single-stranded DNA was prepared from one of these clones and sequenced. The Cyp2e1 cDNA insert was subcloned into the *Xba*I and *Hind*III restriction endonuclease sites of the plagemid vector pTZ18R and 19R, which have reversed polylinker sites, and single-stranded phagemid DNA prepared and sequenced. The DNA sequence, predicted amino acid sequence, sequencing strategy, and restriction endonuclease sites relevant to further experiments involving the Cyp2e1 cDNA are shown in Figure 5.1a, 5.1b and 5.2.

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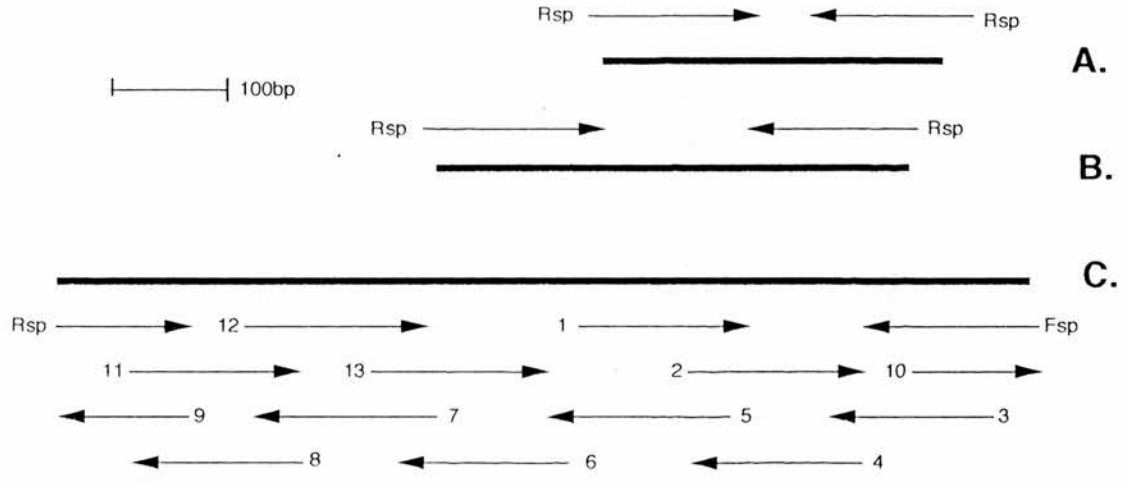


Figure 5.1b: The sequencing strategy and oligonucleotides employed to characterise the 600bp (**A**), 800bp (**B**) and full-length (**C**) Cyp2e1 cDNA clones, and the relative positions of the partial clones to the whole cDNA sequence.

Oligonucleotide sequences

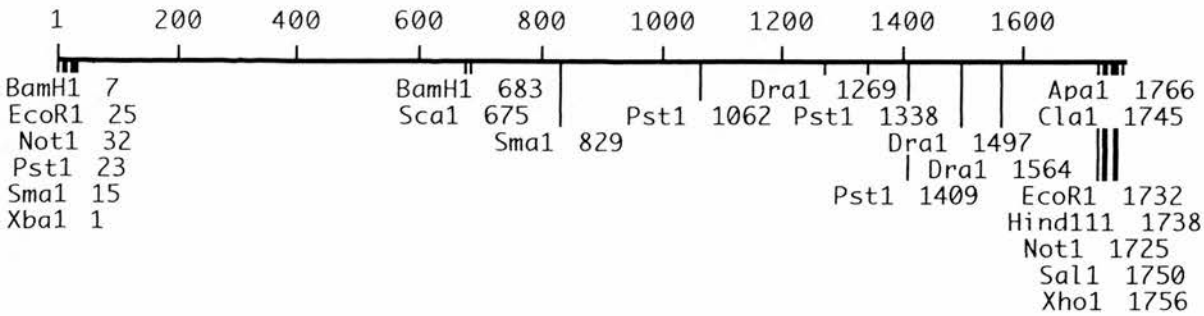
RSP: Reverse sequencing primer

1: GACTTTGGCCGACCTGT
2: GGACTCCCTTTTATTTG
3: GATTGAACTAAGGACTT
4: AAAGCCAATTGTAACAG
5: ATAGTTGTCAAATAAAA
6: TTCTGGGTATTTCATGA
7: TTGGCTTTTCCAAGTGT

FSP: Forward sequencing primer

8: ACATTGCAGGGTGCACA
9: AACACAGGAATGTCCCC
10: CCCACGCGAATTCGTAA
11: ATGAGTTCTCTGGCCGA
12: ATGAGTTCTCTGGCCGA
13: CTGCAATGTCATTGCGG

A.



B.

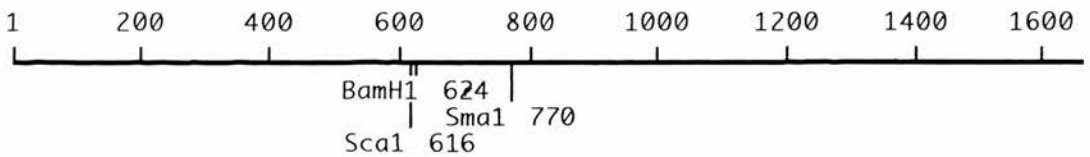


Figure 5.2: The restriction endonuclease sites present in the Cyp2e1 cDNA and the polylinker region of the pBS SK II (-) phagemid. **A:** The restriction endonuclease sites present in the polylinker region of the pBS SK II (-) phagemid and the presence of those sites in the Cyp2e1 cDNA indicating the sites available for cloning strategies. Also present are the restriction endonuclease sites used to orientate the Cyp2e1 cDNA when non-directionally cloned. **B:** The restriction endonuclease sites used to orientate the Cyp2e1 cDNA when non-directionally cloned displayed in isolation of the polylinker sites.

Section 5.2.2: The mouse Cyp2e1 cDNA

On sequencing a Cyp2e1 cDNA insert obtained from the λZAP II library, it was confirmed that the clone contained the complete coding sequence (Figure 5.1a). The Cyp2e1 cDNA encoded a protein with 493 amino-acid residues and a M_r of 56,781. The mouse cDNA displayed a very high level of homology to the CYP2E cDNA sequences obtained from other species suggesting that the function and reactions catalysed by this group of proteins may be highly conserved (Table 5.1).

Table 5.1: The level of sequence homology between the mouse Cyp2e1 cDNA and predicted amino acid sequence and the CYP2E subfamily members characterised in other species. The sequences were aligned using the GCG package Best program (Devereux *et al.*, 1984).

	Percentage identity			
Species	Coding region	Total sequence	Predicted Amino-acid	Reference
Human	79%	75%	77%	Song *et al.*, 1986
Rabbit 1	80%	75%	79%	Khani *et al.*, 1988
Rabbit 2	79%	74%	79%	Khani *et al.*, 1988
Rat	92%	88%	92%	Song *et al.*, 1986

Section 5.2.3: Analysis of the distribution of amino-acid changes between the mouse Cyp2e1 predicted amino acid sequence and those of other members of the CYP2E subfamily

The level of amino-acid homology, or orthology (implying that the similarity seen is evolutionarily related), can be displayed using dot-matrices using generated by a computer program (Genejockey, Biosoft). The amino-acid residues of the two sequences to be compared are written as rows on two axes and a dot is generated on the matrix where the residues are coincident as well as randomly present throughout the other sequence. Using this approach to compare the two rabbit, human and rat CYP2E predicted amino-acid sequences with the mouse Cyp2e1 sequence it can be seen that not only are the sequences highly related, generating a near perfect diagonal amino-acid coincidence, but that the differences that occur between the sequences are scattered throughout the length of the protein. This suggests that the CYP2E subfamily will probably possess highly conserved behaviour as no significant coincidental areas of sequence dissimilarity are present between them (Figure 5.3).

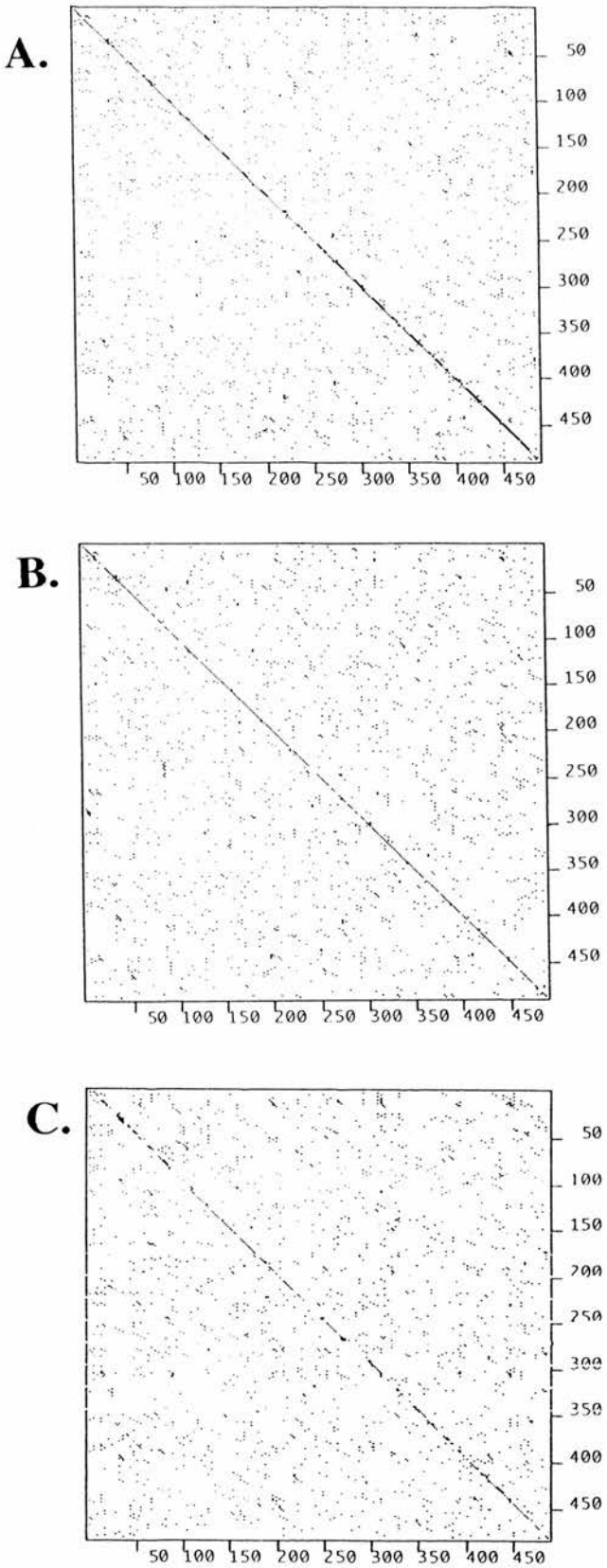


Figure 5.3: The distribution of amino acid changes across the length of the mouse, rat and human CYP2E subfamily protein sequences. In each instance the protein sequences are aligned using the GeneJockey (Biosoft) package and the coincidence of an amino acid residue marked as a point on the matrix. **A:** A comparison of the mouse and rat CYP2E subfamily sequences. **B:** A comparison of the mouse and human CYP2E subfamily sequences. **C:** A comparison of the mouse Cyp2e1 and rabbit CYP2C5 aminio acid sequences (See Figure 5.5).

Section 5.3: Predicting structural and substrate binding features of Cyp2e1 and the CYP2E subfamily

Cytochrome P-450 are ubiquitous in nature and catalyse reactions with the common theme of the insertion of molecular oxygen into a hydrophobic substrate; they possess however a massive variety of substrates which they can metabolise dependent on the particular isoform of the P-450 concerned. Arguably therefore the evolution of P-450 will maintain a tertiary structure which contains the components and features which allow the common-theme reaction to proceed but may modify those residues involved in the substrate interactions of a particular isoform to facilitate the metabolism of the wide variety of chemicals metabolised. If this is the case, the comparison of the predicted amino-acid sequence of Cyp2e1, in conjunction with the highly conserved predicted amino-acid sequences of CYP2E subfamily from other species, to a P-450 with a known tertiary structure, may allow the prediction of regions within the CYP2E subfamily proteins which may play a role both in the generation of tertiary structure and substrate interaction.

The tertiary structure for the bacterial P-450 CYP101 (P-450 CAM) has been resolved and this could form the basis for this analysis. Bacterial P-450 are soluble, unlike the corresponding proteins in eucaryotic cells which are membrane bound; the premise that the overall structure however will be similar in order to maintain the general function may apply and it has been suggested that both soluble and membrane bound P-450 possess overall similar tertiary folding patterns (Zvelebil *et al.*, 1991; Laughton *et al.*, 1990). The N-terminal hydrophobic region found in eucaryotic P-450 may solely account for the differences in the solubility of eucaryotic and procaryotic P-450 proteins; heterologous expression studies have shown that removal of this N-terminal hydrophobic region from eucaryotic proteins has no effect on their activity suggesting that this portion of the protein plays no function role in the reactions of the eucaryotic protein (Cullin & Pompon, 1988; Larson *et al.*, 1991). Most studies now postulate that the N-terminal region of approximately the first forty-five residues acts as a membrane anchor and targets the proteins to the endoplasmic-reticulum. Various models have been suggested for the structure of the N-terminal region with either a single membrane spanning or a hairpin double spanning motif holding the protein in place. Using these models it would be predicted that the rest of the eucaryotic P-450 molecule within the cytoplasm would potentially be folded in a similar manner to its soluble procaryotic counterpart (Nelson & Strobel, 1988; Brown & Black, 1989; Edwards *et al.*, 1989; Kemper & Szczesna-Skorupa, 1989.). Other models however have been proposed for the structure of eucaryotic P-450 suggesting the existence of many membrane spanning regions which would not be consistent with the postulate of a conserved structure

between the procaryotic and eucaryotic P-450 proteins (Mornier *et al.*, 1988; Ozols *et al.*, 1985).

The postulate that procaryotic and eucaryotic proteins with conserved functions will also possess conserved structural features has been successfully demonstrated in connection with several other enzymes. For example, both glyceraldehyde phosphate dehydrogenase (Blesecker *et al.*, 1977) and dihydrofolate reductase (Voltz *et al.*, 1982) on crystallisation have been seen to possess common tertiary structures for example. The comparison of the Cyp2e1 predicted amino acid sequence and P-450cam was therefore undertaken to attempt to assign possible structural and functional regions to the protein.

Section 5.3.1: The structure of P-450cam (CYP101)

P-450cam (CYP101) is a camphor hydroxylase enzyme isolated from *Pseudomonas putida* and its structure has been solved crystallographically both in the presence and absence of its substrate camphor (Poulos *et al.*, 1985; Poulos *et al.*, 1987). A representation of the results gained from this analysis is shown in Figure 5.4. In general terms the P-450 structure broadly divides into a helix-turn-helix rich side and a helix poor side containing the majority of the anti-parallel β-pairs (right and left hand side of figure 5.4 respectively).

Section 5.3.2: The nature of the P-450cam substrate binding site and haem environment

The haem prosthetic group in P-450cam is held between the helices designated L and I in Figure 5.4; the haem proprionate groups are surrounded by hydrogen bond donor residues derived mainly from amino-acids associated with the C-(gln 108, arg 112), K-(asp 297, arg 299) and L-(his 355) helices. An anti-parallel region between phe 350 and gln 360 forms a pocket at the end of the L-helix which houses the cysteine residue, cys 357, which forms the thiolate ligand with the haem. The haem itself provides the largest hydrophobic surface within the molecule for interactions with the substrate, however amino-acids associated with the F-helix (thr 185), I-helix (leu 244, val 247), the K-helix (val 295, asp 297) and the β ' anti-parallel region (phe 87, tyr 96) are also seen to interact with the camphor molecule (Poulos *et al.*, 1987). How camphor enters the molecule is unclear and unlike the structures of many other enzymes, P-450cam does not possess an active site defined as an open cleft but, due to the hydrophobic nature of its substrate has an active site sequestered within the interstices of the molecule.

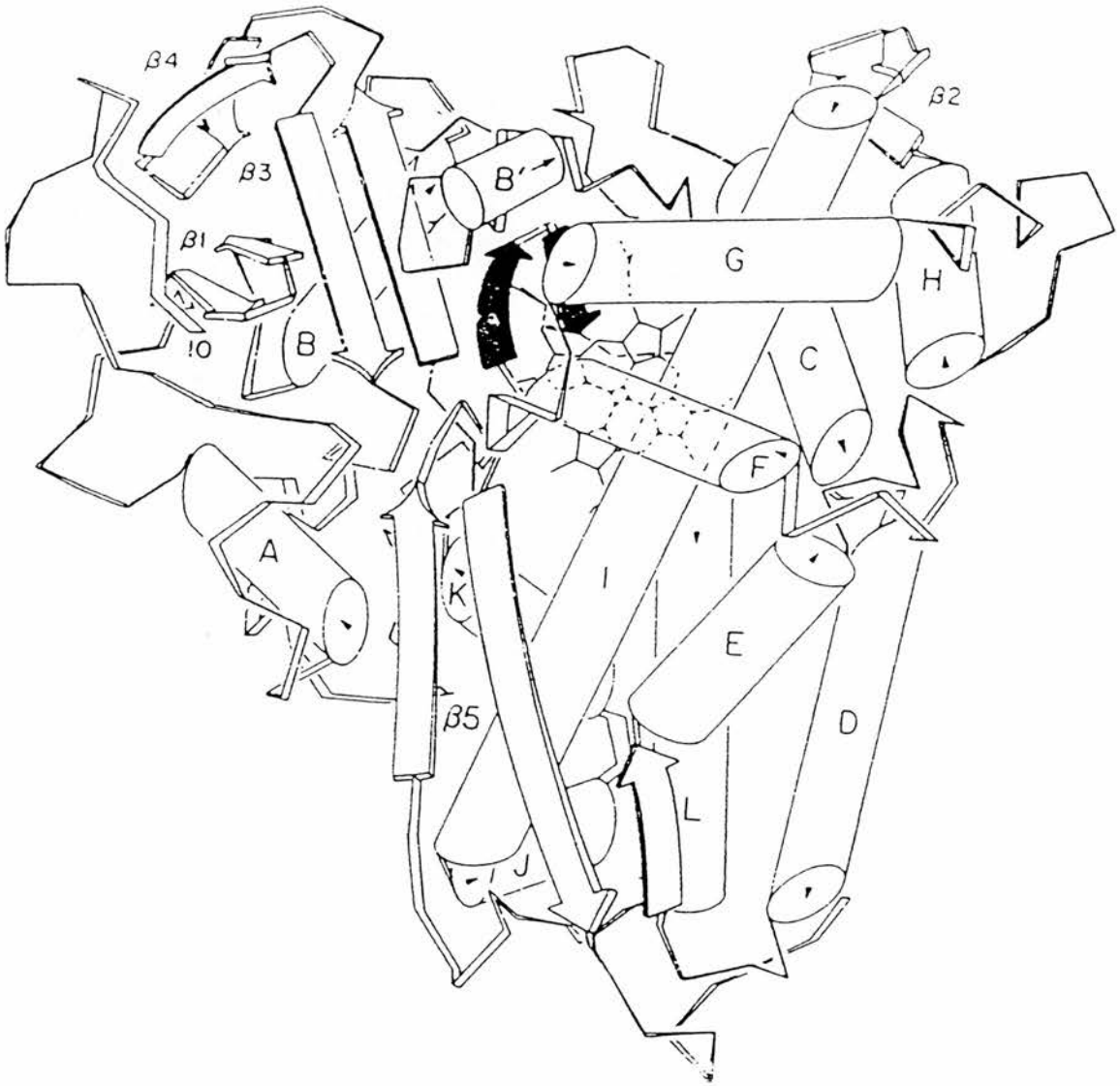


Figure 5.4: A representation of the high resolution structure of the soluble bacterial CYP101 (P450cam) protein structure and the protohaem group. The nomenclature for α-helices and β-sheets in this diagram are those used in the text. Figure modified from Poulos, *et al.* (1987).

It is possible that the camphor molecule enters the P-450 through an access channel bounded by hydrophobic residues which opens and shuts with the "breathing" of the structure. The β3 region of the P-450 molecule, just outside the K-helix and adjacent to the β' region containing the haem interacting residues is suggested to play an important role in determining the ability of the P-450 molecule to accommodate its substrate (Figure 5.4). Oxygen co-ordinates with the iron atom and so the portions of the I-helix which have been seen to interact with the haem may interact with the oxygen molecule also. The conservation of primary amino-acid sequence around this area between a wide variety of functionally diverse P-450 is striking and strengthens the suggestion that the proteins may all possess a similar structure based around the need to perform a common enzymatic step (Nelson & Strobel, 1988). In this context most P-450 possess either a glycine or an alanine at the residue equivalent to gly 248 in the P-450cam I-helix as well as a highly conserved threonine, thr 252 in P-450cam, residue close by. The P-450cam I-helix containing these features is, at thirty-four residues, relatively long and all the residues it contains possess normal helical hydrogen bonding with the exception of thr 252. Thr 252 generates a kink within the I-helix in the crystal structure; the generation of the I-helix kink is thought to allow the formation of a pocket which may accommodate the oxygen molecule and this pocket is further stabilised by a complex series of hydrogen bonds which form around the kink. It is thought that this potentially conserved pocket is capable of restricting the relative positioning of the oxygen and camphor adjacent to the haem moiety within the P-450cam molecule and so the nature of the products generated (Poulos *et al.*, 1987).

Section 5.3.3: The P-450 superfamily: a group of enzymes with structures capable of evolving rapid changes in substrate specificity

If all P-450 adhere to a similar helically determined tertiary skeleton as seen in P-450cam, it is possible that the delineation of the P-450 enzyme into a structurally rigid helix-rich moiety and a less well defined globular moiety has not occurred by chance. The rigid portion of the structure appears to make the principal interactions with the haem and generates a pocket within the I-helix for oxygen binding and orientation that will be required by all P-450 isoforms as they all catalyse the same common theme reaction. As a result of the structural restraints generated by the common reaction requirements it would be expected that these regions of the P-450 molecule would be most highly conserved. Around these conserved structurally restrained regions however, it would be possible to generate evolutionary modifications in the less structured moiety of the molecule and the intervening loops between structural elements. The P-450 molecule may therefore represent a molecule in two structural

parts, one part the haem and oxygen binding portion providing the engine to drive the common reaction and thus possessing a highly conserved structure, the other part determining what substrates the molecule is capable of accommodating and metabolising which is consequently less structurally restrained.
The specificity of the accommodation could be determined at two levels. Firstly modification of residues at the substrate entrance site could determine the chemicals which can gain access to the haem and oxygen associated reaction centre, and secondly, having once entered the molecule, the location of other critical residues would determine those molecules capable of being held in a productive orientation relative to the active site. The nature of the residues contributing to the generation of these two theoretical levels of substrate specificity could be changed not only by direct modification of the key interacting residues themselves, but also more subtly through the modification of other more distal residues leading to perturbation of the enzyme structure as a whole. As a result of this, although it may theoretically be possible to predict those portions of a P-450 sequence that contribute to the conserved structural part of the molecule, the prediction of residues that may be responsible for the generation of substrate specificity would be expected to be much more difficult.

Section 5.3.4: Comparing the CYP2E subfamily to P-450cam (CYP101)

By aligning the predicted amino-acid sequences of the known CYP2E subfamily members and generating a consensus which is then aligned to that of CYP101 (P-450cam), an idea of those portions contributing to the structural and functional regions of the CYP2E subfamily can potentially be obtained. Refinements to this general procedure have been obtained in the modelling of other P-450 sequences to CYP101 by shifting the alignment generated to take into account conserved structural requirements of the molecules, such as haem contacts, as well as the insertion of secondary structures predicted to be generated by certain amino-acid groups by analogy to other known structures outside expected structural domains of the P-450 molecule. In this manner, 3-D models of eucaryotic P-450 structures have been generated (Zvelebil, *et al.*, 1991; Laughton *et al.*, 1990). A linear comparison of the known CYP2E subfamily sequences and P-450cam is shown in Figure 5.5; predicted structural regions of the CYP2E subfamily as a consequence of this alignment are marked. The incorporation of the rabbit CYP2C5 amino-acid sequence into the CYP2E subfamily alignment exploits the energetic predictions previously modelled into a structural prediction of mammalian P-450 (Zvelebil *et al.*, 1991).

Figure 5.5: An alignment of the CYP2E subfamily, rabbit CYP2C5, and CYP101 (P450cam) amino acid sequences. The predicted amino acid sequences for all the CYP2E subfamily members isolated to date were aligned with the mouse Cyp2e1 predicted amino acid sequences using the GCG package Best and Lineup programs. Only at points where amino acid residues differ from the Cyp2e1 sequence are they displayed; a consensus for the CYP2E subfamily protein sequence was determined from this information as is displayed below the aligned CYP2E subfamily sequences. The CYP2E consensus sequence was aligned with the rabbit CYP2C5 protein sequence which had previously been compared to CYP101 (P450cam) (Zvelebil, *et al.* , 1991) allowing a comparison of the CYP2E subfamily sequences and the crystallographically solved CYP101 protein to be undertaken. The structural features of CYP101 are boxed and annotated according to the nomenclature of Poulos, *et al.* (1987) (See Figure 5.4). Boxed areas of the CYP2E subfamily correspond to those areas predicted to interact with the haem group (**H**) and substrates (**S**) and are discussed in the text. The following features of the alignment, also discussed in the text, are also marked:

1) The exon arrangement of the CYP2E subfamily, lined below the consensus sequence.
2) The potentially phosphorylated serine group in the C-helix (*).
3) The kinked region of the I-helix suggested to hold the substrate and oxygen in apposistion (**S & Ox.**).
4) The potential phosphorylation site in CYP101 (**CYP101 Phos. site**).
5) The pocket in the P-450 structure suggested to form a pocket enclosing the haem (**Haem Pocket**).

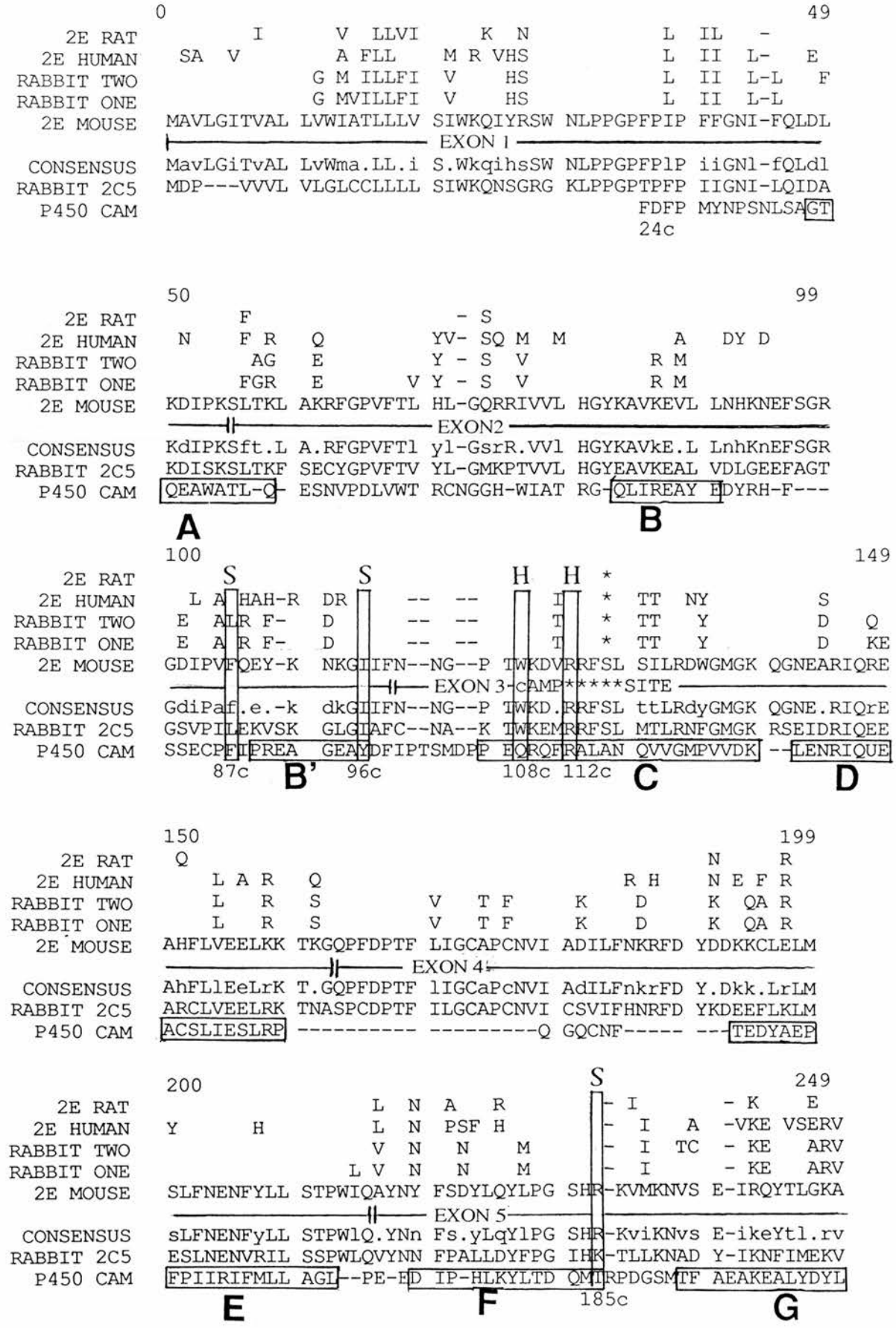


Figure 5.5 (continued)

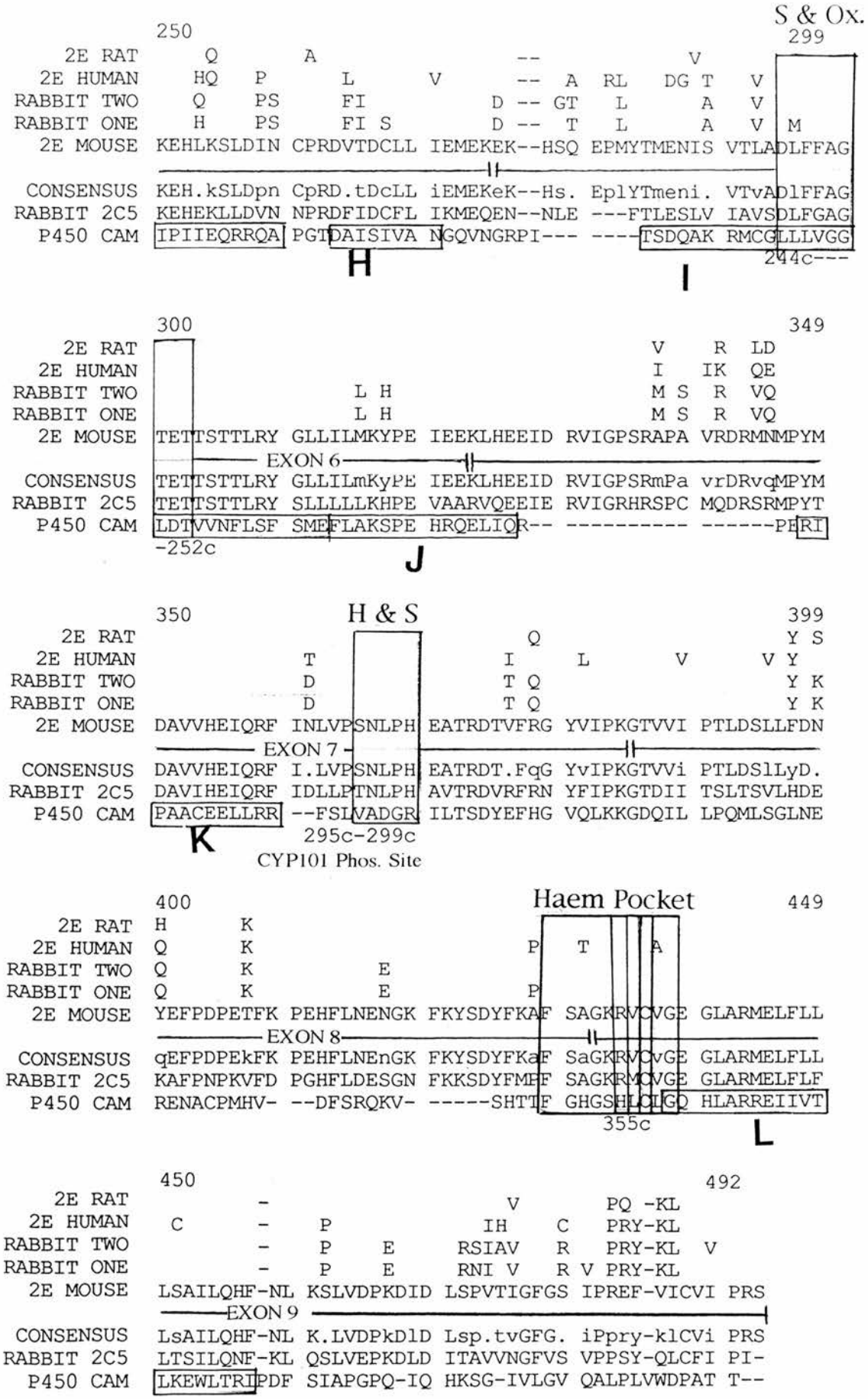


Figure 5.5 (continued)

Section 5.3.5: Predicted features of the Cyp2e1 protein

The haem of P-450cam is held between the L- and I-helices (Figure 5.4); equivalently placed amino-acids in the Cyp2e1 protein and the CYP2E subfamily in general are shown in Table 5.2 and Figure 5.5 respectively.

Table 5.2: The amino acids seen to interact with the haem group in P-450cam and the predicted cognate residues in Cyp2e1

Nearest structural region	P-450cam residue	Predicted Cyp2e1 equivalent residue
C-Helix	Gln108	Trp122
	Arg112	Arg 126
K-helix	Asp 297	Leu 367
	Arg 299	His 369
L-helix	His 355	Arg 434

Three of these five predicted Cyp2e1 haem contacts could theoretically form hydrogen bonds analogous to those present in P-450cam: arg 112/ arg 126, arg 299/his 369, and his 355/ arg 434. These three amino-acid residues are invariant in the aligned CYP2E subfamily sequences (Figure 5.5) and are also seen to be highly conserved in multiple alignments carried out on a large number of mammalian P-450 (Nelson & Strobel, 1988; Nelson & Strobel, 1989). Equivalent hydrogen bonds could not however be provided by the Cyp2e1 leu 364 or trp 122 residues.

The I-helix of P-450cam, with the thr 252 generated kinked oxygen pocket, is potentially seen to be preserved in the thr 252 of Cyp2e1 which is also surrounded by several other proportionately spaced similar amino acid residues. This portion of the P-450 structure has been seen to be highly conserved in multiple alignments of several P-450 isoforms, suggesting the potential validity both of the suggested functional role of this feature of the molecule and also that it forms a highly conserved structural motif in all P-450 molecules (Poulos *et al.*, 1987). The presence of several other threonine residues around this proposed oxygen pocket in Cyp2e1 may potentially increase the size of the kink in the I-helix and affect the substrates which it is capable of metabolising.

The predicted C-region of the Cyp2e1 protein, in common with many other family 2 P-450, is seen to contain a potentially phosphorylatable serine 129 residue within a canonical basic kinase recognition site. The potential relevance of this and the effect of

introducing such a post-translational change in a region of the protein structure predicted to form contacts with the haem are discussed in more detail in Section 4.7.3.
As in all P-450 studied to date, a cysteine forming the thiolate axial ligand between the P-450 protein and the haem group is seen; in P-450cam this is cys 357 whose equivalent residue is predicted to be cys 435 in CYP2E subfamily. The thiolate binding cys 357 in P-450cam is held within a β-bulge pocket bounded by phe 350 and gly 359 whose equivalent residues in CYP2E may be phe 428 and gly 437 respectively.
All these features and their relative positioning to predicted structural elements of P450cam are illustrated in Figure 5.5.

Section 5.3.6: Amino-acids potentially contributing to the substrate binding site of CYP2E subfamily

From the co-crystal derived from P-450cam and its substrate camphor the principal amino-acids making contacts with the substrate were ascertained (Poulos *et al.*, 1985). Using the structural alignment it may be possible to predict the equivalent residues which may interact with the CYP2E subfamily substrates. Those residues seen to be making both major and minor contacts with camphor in P-450cam and their equivalent residues in CYP2E subfamily are shown in Tables 5.3a and 5.3b.

Table 5.3a: Amino acids seen to make major contacts in P-450cam with its substrate camphor and their equivalent residues on alignment with the CYP2E subfamily.

Camphor/P-450cam interacting residue	Closest structural element	Equivalent residue in the CYP2E subfamily
Phe 87	β'-helix	Phe 108 (Rabbit 2,Leu 108)
Tyr 96	β'-helix	Ile 114
Leu 244	I-helix	Asp 296

Table 5.3b: Amino acids seen to make minor contacts in P-450cam with its substrate camphor and their equivalent residues on alignment with CYP2E subfamily.

Camphor/P-450cam interacting residue	Closest structural element	Equivalent residue in the CYP2E subfamily
Thr 185	F-helix	Arg 233
Val 247	I-helix	Phe 298
Val 295	β_3-region	Ser 366
Asp297	β_3-region	Leu 368

The putative CYP2E subfamily substrate interacting residues are highly conserved between species suggesting that the proteins may be expected to display a high degree of similarity in the substrates which they metabolise. The one exception to this is the replacement of the consensus phe 108 by leu 108 in the rabbit CYP2E2 protein. The change of this residue would be predicted to occur within the β'-helix/β3-antiparallel region adjacent to both the axial haem cysteine ligand and the oxygen encompassing kink in the I-helix in the rabbit CYP2E 2. Such a change could potentially be expected to affect the substrates metabolised by this isoform or its general kinetic parameters; enzymatic analysis of both of the purified rabbit isoforms demonstrated that CYP2E2 was less active in the metabolism of nitrosamines, ethanol, aniline and chloroxazone (Pernecky *et al.*, 1991). It is clear however that, with reference to the proposal of the P-450 structure as being composed of both a rigid and more plastic moiety, other regions and residues of the protein may play an important role in the generation of substrate specificity of a specific isoform.

Section 5.3.7: The validity of structure and function predictions of membrane bound P-450 based on structural evidence from the soluble P-450cam

The validity of this kind of structural and functional prediction of membrane bound mammalian P-450 based on information gained from P-450cam can not be truly assessed until the crystal structures of other P-450 have been obtained. In the past the alignment of families of sequences and the application of algorithms predicting structures on the basis of known structural motifs have proven to be successful as seen, for example, in the case of tryptophan synthase (Crawford *et al.*, 1987).
Site directed mutagenic studies conducted on those regions of the P-450 protein predicted, as a result of similar alignment studies, to be involved in substrate metabolism have proved successful, suggesting that the hypothesis that the P-450

retains an overall conserved structural topology is valid. For example, mutant P-450 proteins generated with modifications in the size of the predicted I-helix oxygen kink can modify the kinetics of the resultant enzyme; the replacement of the proline, that generates the kink, by valine in the rat CYP1A2 protein greatly reduced the enzymic activity towards benzephetamine and 7-ethoxycoumarin (Furuya *et al.*, 1989), and similar results were seen on the removal of the equivalent kink inducing residue in the human aromatase CYP19A1 in its subsequent activity towards androgens (Graham-Lorence *et al.*, 1991).

Mutagenesis of residues in the β'-helix/β3-antiparallel region, predicted to be important in the determination of enzyme specificity and adjacent to both the axial haem cysteine ligand and the oxygen encompassing kink in the I-helix, have been seen to affect the substrates metabolised and the kinetics of the reactions, adding validity to an argument for a conserved structural/functional role for this portion of the molecule (Kronbach *et al.*, 1989; Uno & Imai, 1987; Aoyama *et al.*, 1989).

The concept that the substrate specificity of a P-450 enzyme can be dramatically modified by changing a single amino-acid is well illustrated by experiments on the mouse Cyp2a4 and Cyp2a5 proteins. Although these proteins differ only in eleven amino-acid residues dispersed throughout the enzyme they have greatly divergent substrate profiles; Cyp2a4 metabolises, amongst other things, testosterone, and Cyp2a5, coumarin. By changing the eleven differing amino-acids in these two proteins it was seen that some were critical in determining the specificity of each isoform for either coumarin or testosterone. If phe 209 was modified in Cyp2a5 to a leu, as present in Cyp2a4, the specificity of the enzyme was changed from coumarin to testosterone. This mutation would be predicted to modify the structure of the P-450 between the F- and G-helices near to the regions seen to interact with the substrate in P-450cam and suggested to be important in the determination of those molecules which the P-450 structure can accommodate. Other residues which were seen to be important in the determination of Cyp2a4/5 specificity, map to the β3 region of the molecule adjacent to both the haem and I-helix oxygen pocket (Lindberg & Negishi, 1989; Gotoh, 1992).

The results from these studies go some way to validate the suggestion that all P-450 may have a conserved structure evolving through the modification of residues at key sites. The true validity of this suggestion however can only be gauged when further P-450 crystals are available allowing structural comparisons to be drawn and potential regions of conservation to be reinforced. There are reports of two new crystal structures which are being prepared which may enable such a comparison to be established. The two structures are those of the bacterial P-450terp, and the P-450 portion of the catalytically self-sufficient bacterial P-450BM-3 (CYP102) enzymes; results obtained from these crystals may allow the reappraisal of the value of structural

and functional predictions made for mammalian P-450 on the basis of the extrapolation of data gathered from P-450cam (Petersen *et al.*, 1992).

Section 5.4: Features of the untranslated regions (UTRs) of the Cyp2e1 message

Section 5.4.1: Disruption of the Cyp2e1 3' UTR by a β2 repeat element

The 3' untranslated region (3' UTR) of the Cyp2e1 message has become disrupted sometime after the speciation of mice by a β2 repetitive element; as a result of this disruption the poly-(A) addition site used by the Cyp2e1 mRNA is that of the β2 repetitive element itself. The β2 element present in the 3' UTR of the Cyp2e1 message was completely sequenced from the Cyp2e1 gene as the cDNA clones terminated before the end of the repeat element. The β2 element is highly similar to those observed previously in other messages and genes and possesses the characteristic repeat motifs at the 3' and 5' boundaries of its insertion site (Figure 5.6).
The origin, function and mechanism of insertion of such elements are still unclear but it appears that they form a family of such motifs present in many organisms. The best studied of this family of repeating elements is the human "*Alu* " element to which the mouse β elements may be related. The biology and function of these elements, as well as the implication of the presence of such a feature in the Cyp2e1 mRNA, are discussed.

Section 5.4.2: Repetitive transposable elements

S1 nuclease digestion of rapidly denatured and reannealed genomic DNA revealed the presence of rapidly annealing repeating elements resistant to nuclease attack in the genomes of many organisms; in mammalian genomes these segments of DNA represent a series of retro-transposable repetitive elements. This group of repetitive sequences divides into two subpopulations based on size; firstly smaller elements, termed short interspersed repeat elements (SINEs) and secondly larger elements, Large interspersed repeat elements (LINEs); LINES they are suggested to be of a different evolutionary origin from SINES and are not further discussed here (Jelinek & Schmid, 1982; Jelinek *et al.*, 1980; Deninger *et al.*, 1992).
On sequencing, both rodent and human SINEs were seen to fall into two main groups by homology; firstly the primate *Alu* and the homologous rodent β1 family and secondly the rodent β2 family. SINEs from both families are seen to be present in both orientations throughout the rodent genome and are seen to be transcribed either due to their insertion into DNA dependent RNA polymerase I or II (pol I and II)

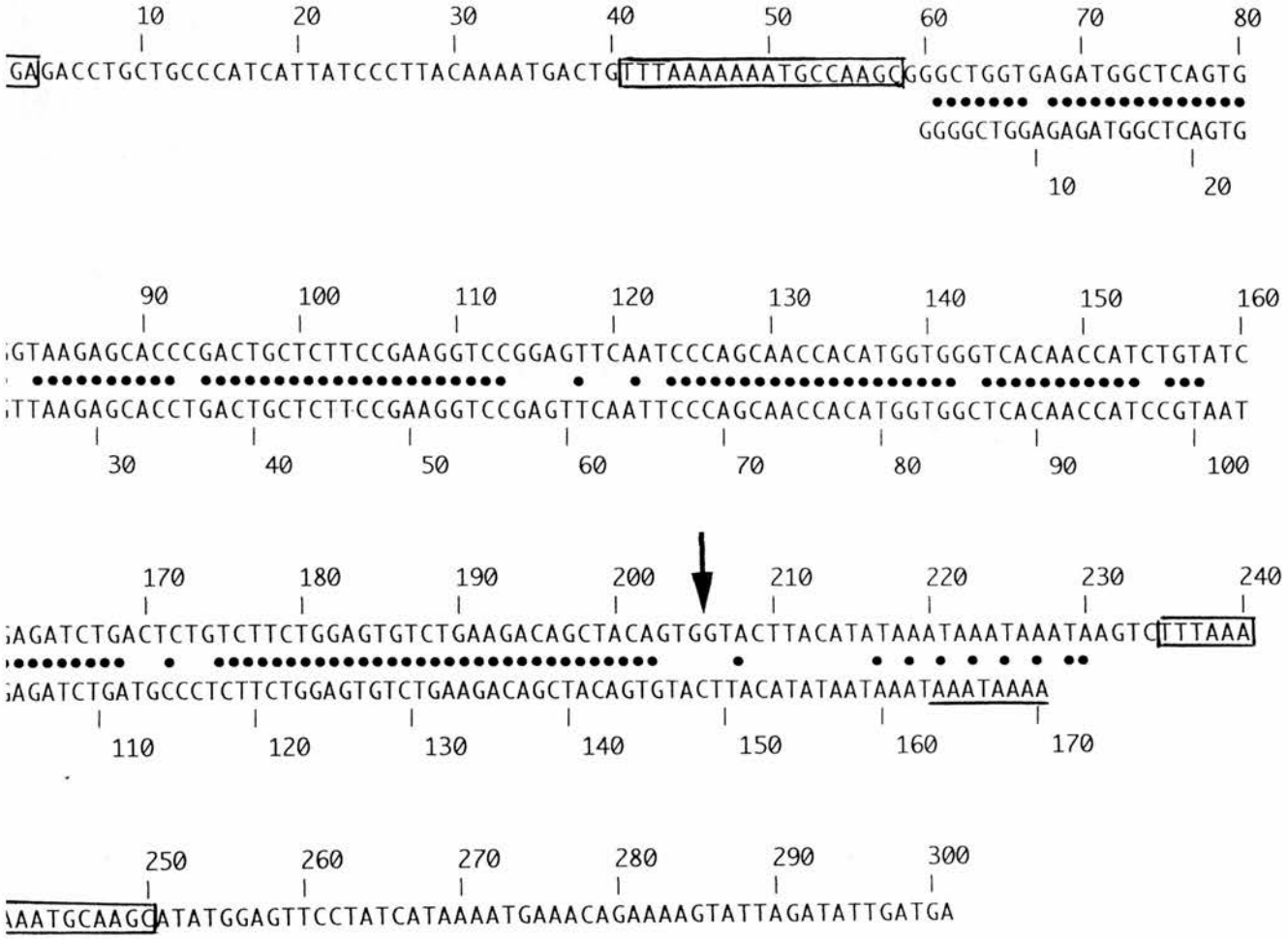


Figure 5.6: Alignment of the 3 ' UTR of the Cyp2e1 transcript and the rodent β2-repeat element. The mouse Cyp2e1 sequence (upper) and the rodent β2-repeat element (lower) (Krayev, *et al.* , 1982). The stop codon of the Cyp2e1 mRNA is boxed as are the repeat sequence boundary motiffs which probably represent the site of insertion of the β2-repeat element. The poly-A tail addition site in the β2-repeat element is underlined. The arrow marks the end of the sequence present in the cDNA clone and data beyond this is compiled from genomic sequence (See Section 5.8.3)

transcriptional units, or as the result of transcriptional initiation from the internal DNA dependent RNA polymerase III (pol III) promoters present within some of the SINEs themselves; however, no open reading frame is found in the SINEs (Rogers, 1985; Krayev *et al.*, 1982; Kramerov *et al.*, 1979).

The best studied of these elements is the human *Alu* family, so termed because the repeating element contains an *Alu* I restriction endonuclease recognition site. The primate SINE *Alu* is repeated at between three and five million copies in the genome and is around three hundred base-pairs in length with around a ten percent sequence variation amongst primates, suggesting a neutral drift of sequence retention between species in these non-coding elements (Houck *et al.*, 1979; Deinger *et al.*, 1981). The rodent β1 family is around one hundred and thirty base-pairs in length, is thought to exist at between ten and fifty thousand copies in the rodent genome and is related to the primate *Alu* SINE. The *Alu* and β1 SINEs appear to have been generated as the result of retrotransposition of a previously functional structural RNA molecule. Both *Alu* and β1 SINEs show a high degree of sequence similarity to the 7SL RNA molecule which is a structural component in the signal recognition particle (SRP) involved in the translocation of proteins across the lumen of the endoplasmic reticulum. As such both *Alu* and β1 SINES are regarded as processed retro-pseudo genes (Elder *et al.*, 1981; Walter & Blobel, 1982). The primate *Alu* SINE is generated essentially from a dimer of two such 7SL elements which is suggested to have been the result of recombination of one 7SL retrotransposon into the A-rich 3' end of another such element already in place in the genome; the rodent β1 element however is a monomeric 7SL-like sequence. In both *Alu* and β1 SINEs there is an internal deletion of around one hundred and fifty nucleotides from the 7SL molecule and it is suggested that this may be the result of reverse transcription activity missing out a highly structured region of the 7SL molecule; it is possible that the loss of this portion of the 7SL has resulted in the release of the element from functional restraints, leading to sequence drift from its 7SL progenitor (Walter & Blobel, 1982; Weiner *et al.*, 1986). The right hand element in the *Alu* dimer has independent transcriptional activity but the left hand end of the dimer no longer possesses a functional pol III promoter and is therefore transcribed only in association with the left hand component. The rodent β1 SINE family is more like the right hand component of the primate *Alu* dimer as only one functional pol III promoter has been reported in this family (Haynes *et al.*, 1981; Weiner *et al.*, 1986).

The β2 family of SINEs is thought to have arisen through retrotransposistion of a previously functional RNA element by a similar mechanism to that proposed for the generation of the *Alu* and β1 SINEs. The progenitor molecule for the retrotransposistional event is thought to be a tRNA molecule (Laurence *et al.*, 1985). The homology between the active β2 SINE pol III promoter and that present in tRNAs

is high but the sequence drift in the β2 SINE as a result of loss of structural constraints has resulted in too great a divergence to allow a precise determination of the parental tRNA molecule (Laurence *et al.*, 1985). Unlike the β1 and *Alu* SINE families the β2 SINE family contains a functional poly-(A) addition signal (Krayev *et al.*, 1982).

The exact series of events which lead to retrotransposistion and proliferation of these elements is obscure. It is suggested that structural RNA derived SINEs may be particularly prone to self-priming; thus the element, generated either as a discreet RNA pol III transcriptional unit or in conjunction with another message, by some mechanism generates a reverse transcribed copy, second strand synthesis and then recombines back into the genome (Jagardeeswaran *et al.*, 1981; Flavell, 1981; Baltimore, 1985). Retrotransposition of such elements has been seen to lead to the disruption of functional genes, as in the case of the loss of a functional neurofibromatosis type I associated gene following *Alu* SINE insertion (Wallace *et al.*, 1991). A β2 element has been seen to have inserted into one of the 18S rRNA genes in a mouse cell line leading to the loss of the functional transcript; this insertion is not present in the parental mouse cell line, suggesting that the insertional event has occurred within the past decade (Oberbaumer, 1992). Tissue culture systems selecting for the loss of a negative selection marker by *Alu* SINE disruption have been developed to illustrate retrotranspositional events occurring; in this instance retrotransposition was accelerated by ultra-violet radiation potentially increasing genome damage and recombination (Linn *et al.*, 1988; Linn *et al.*, 1989).

The insertion of a SINE element also increases the recombinational activity around the region of the genome associated with its insertion site; thus SINE elements have been seen to cluster, with one element inserting into another making this region of the genome unstable as seen in the case of *Alu* driven recombinational events in the human genome (Lehrman *et al.*, 1987; Rouger *et al.*, 1987) and the clustering of β2 elements within the rat growth hormone gene (Barta *et al.*, 1981; Page *et al.*, 1981).

SINE insertion can result in the loss of a transcriptionally functional unit either through the subsequent generation of an incorrect message, inhibition of correct splicing, or the generation of a recombinational hotspot and the loss of that portion of the genome through recombination between sequences containing SINE elements (Lehrman *et al.*, 1987; Rouger *et al.*, 1987).

The biological function, if any, of these families of sequences is unclear. The level of retrotransposistional activity is suggested to become elevated in embryonic and cancerous cells as well as in situations which result in genomic damage leading to the suggestion that these elements may play a role in the repair and rescue of damaged chromosomes (Linn *et al.*, 1988). The fact that these elements may have their origin in otherwise functional structural RNA molecules however suggests that they may

represent artefacts resulting from a coincidence of aberrant reverse transcriptase and the self-priming activities of the elements and that they may not therefore be performing any directed function within the cell. What is clear however, even if a true role for these elements can not be arrived at, is that the disruption of functional transcriptional elements within the genome, either by direct retrotransposition or subsequent homologous recombinational events, has the potential to generate significant genomic disruption (Wallace *et al.*, 1991; Grossbauer, 1984; Rouger *et al.*, 1987; Lehrman *et al.*, 1987).

Section 5.4.3: The significance of the β2 element in the Cyp2e1 3' UTR

The insertion of the β2 SINE into the 3' UTR of the Cyp2e1 gene means that the mRNA generated no longer contains the endogenous 3' sequence and the message now terminates at a poly-(A) addition site within the β2 SINE. As discussed (Section 5.4) the Cyp2e1 message level becomes elevated in starvation in common with results obtained from studies on the CYP2E subfamily messages of other species; the mechanism suggested to account for this event in the rat has been suggested to be through the stabilisation of pre-existing CYP2E1 message (Song *et al.*, 1987). Previous messages controlled as a result of regulated message stability have been seen to contain features within the non-coding portions of the message, and principally within the 3' UTR generating a structural motif interacting with a regulatable RNA binding protein (Section 3.7.6). Loss of the Cyp2e1 3' UTR as a result of the β2 SINE insertion would therefore theoretically lead to the loss of this form of regulation if it were executed via a conventional 3' UTR-related mechanism. The fact that the elevation of the Cyp2e1 mRNA still occurs in starvation suggests that, unless a protein recognises a motif in the remainder of the 3' UTR, there is no role for this portion of the 3' UTR in this event and either that stabilisation occurs by a previously unknown mechanism, potentially involving sequences within the 5' UTR or motifs within the coding region, or that stabilisation does not actually occur and the elevations observed may be transcriptional (Section 3.7.7). The β2 element insertion into the Cyp2e1 3' UTR in this manner provides a genetic argument against a role for conventional message stabilisational control of the CYP2E mRNA levels.

Another implication of the presence of the β2 SINE within the Cyp2e1 gene is the possibility that the gene may be open to recombinational modification as the result of further retrotransposistional and crossing-over events as seen to occur in *Alu* driven recombination (Lehrman *et al.*, 1987; Rouger *et al.*, 1987). Such an event may already have occurred in an as yet undefined mouse strain potentially leading to the functional disruption of the Cyp2e1 gene.

Section 5.4.4: The nature of the 5' UTR of the CYP2E subfamily messages

The sequences of the known rat and human CYP2E1 5' UTRs and the predicted mouse Cyp2e1 5' UTR are highly conserved. These 5' UTR sequences were analysed to generate the most energetically stable RNA folded structure using the GCG package Stemloop and Fold programs (Devereux *et al.*, 1984; Zucker *et al.*, 1989) and it was seen that a similar folding pattern was generated by all these CYP2E subfamily messages. To establish the accuracy of the predicted 5' UTR folding patterns obtained from these programs the iron responsive element (IRE) of the human ferritin H mRNA (-186 to -143) was also folded using the same parameters and the result compared to the previously characterised and experimentally established structure of this sequence (Rouault *et al.*, 1988). The structure generated by the CYP2E subfamily 5' UTR forms a strong stem-loop structure similar in size to the IRE previously shown to be involved in the regulatable translatability and stability of the ferritin and transferrin receptor messages (Section 3.7.6). The 5' UTR of the CYP2E1 subfamily sequence was seen to contain a consensus $NF_{\kappa\beta}$ recognition site and could potentially be bound by a series of protein factors (Section 4.13). The results of this analysis are shown in Figure 5.7a and Figure 5.7b.

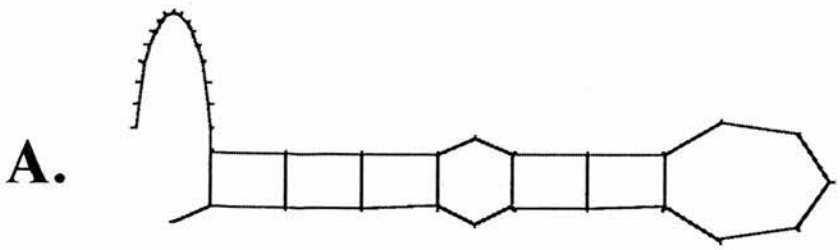
$NF_{\kappa\beta}$ sites are bound by a series of transcription factors including the *rel*-family which includes $NF_{\kappa\beta}$ itself, the development controlling " *dorsal* " factor of *Drosophila* as well as the oncogene product *v-rel* and its cellular counterpart *c-rel* (Lewin *et al.*, 1991) and a rapidly expanding series of unrelated factors such as C/EBP (Clark *et al.*, 1988), MBP-1 (Baldwin *et al.*, 1990), KBF-1 (Israel *et al.*, 1987), and H2TF1 (Baldwin *et al.*, 1987). It appears that as a general rule eucaryotic transcription factor recognition sites can be bound by more than one protein factor in a competitive manner. These protein elements, their nature and possible regulation are discussed in more detail in Section 4.13.1.

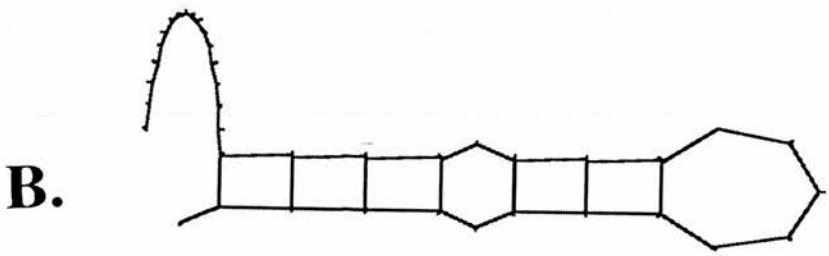
The consensus sequence for $NF_{\kappa\beta}$ type sites is formed by a dimeric pentamer of $GGG^{A}/_{C}TN^{T}/_{C}CC$; within this site the completely conserved residues are GGG(NNNNNN) C where N is a defined series of nucleotides around a limited number of variable residues which may confer specificity to one or more of the variety of factors capable of binding these sites (Figure 5.7b). Purified $NF_{\kappa\beta}$ was seen to be capable of binding single-stranded DNA containing this motif and this was capable of competing with double-stranded DNA for the protein (Zabel *et al.*, 1991). The putative $NF_{\kappa\beta}$ site within the folded 5' UTR of the CYP2E subfamily messages retains a correctly orientated site containing all the completely conserved residues and all but two of the less conserved consensus residues present in the site bound by $NF_{\kappa\beta}$ itself are retained. No thiamine residues are conserved in any of the $NF_{\kappa\beta}$ type recognition sites

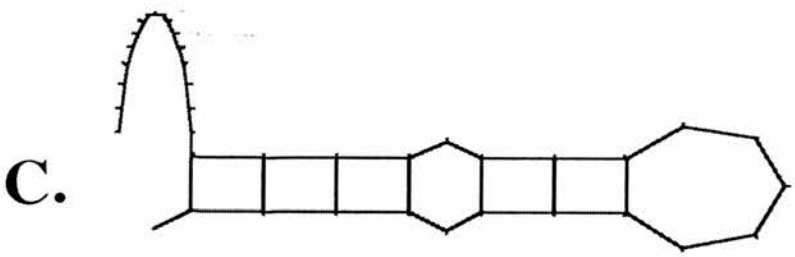
suggesting that the replacement of these nucleotides by uracil in the potential RNA derived recognition site may not affect the binding ability of a protein (Figure 5.7b). Although studies on $NF_{\kappa\beta}$ protein itself have shown that the protein is capable of binding a single stranded recognition site, the folding of this portion of the CYP2E subfamily 5' UTR renders the majority of the potential recognition site double stranded. Interactions between transcription factors and RNA molecules have been previously noted specifically for those proteins which possess zinc-finger binding motifs; zinc-finger proteins are thought to be very ancient and in their ability to bind RNA as well as DNA potentially pre-date a DNA derived genome (Dressler & Gruss, 1988). $NF_{\kappa\beta}$ protein itself requires zinc for binding to its recognition sequence although the factor does not appear to contain any of the previously recognised zinc-finger motifs (Zabel *et al.*, 1991); in the case of other factors capable of binding the $NF_{\kappa\beta}$ site, such as MBP-1 there is sequence evidence for the presence of a consensus $cys_2 \cdot X_{12} \cdot his_2$ zinc-finger motif (Baldwin *et al.*, 1991).

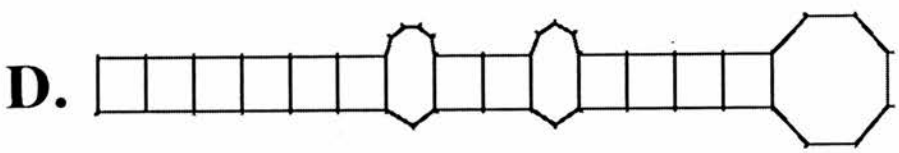
Although clearly speculative the suggestion that the 5' UTR of the CYP2E subfamily messages may contain an $NF_{\kappa\beta}$ recognition sequence within a secondary structural feature generated by a conserved sequence is of interest. Such a feature of the message may allow, in a manner akin to the regulation of translatability of ferretin mRNA (Section 3.7.6), control to be exerted, potentially through a factor like MBP-1, at the translational level on these messages. Such an event could go some way to explaining some of the anomalies which have arisen concerning the regulation of these messages.

Figure 5.7a: The predicted folding pattern adopted by the 5' untranslated regions (UTR) of the mouse, rat and human CYP2E subfamily transcripts. The suboptimal folding patterns of the 5' UTRs of the 3 sequences, from +3 to - 39, were generated using the GCG package Stemloop and Fold programs **A:** Mouse Cyp2e1 5' UTR. **B:** Rat CYP2E1 5' UTR. **C:** Human CYP2E1 5' UTR. **D:** The 5' UTR IRE (iron response element) of the human ferritin H mRNA, from -186 to -143, was also folded using the same parameters as those employed to generate the predicted structures of the CYP2E subfamily and this analysis is shown. **E:** The published suboptimal folding pattern generated for the -186 to -143 region of the ferritin mRNA (taken from Rouault *et al.* , 1988). This region has been shown experimentally to be involved in the regulation of the ferritn mRNA function. The inclusion of **D** and **E** indicates that the folding programs used to generate the CYP2E subfamily predicted structures produce similar results to those used previously in the ferritin 5' UTR and that the stem-loop predicted to be present within the 5' UTR of the CYP2E subfamily is of a similar size to those shown previously to be capable of interacting with mRNA binding proteins.
In each instance the sequences run from 5' (top) to 3' (bottom); in the CYP2E subfamily sequences the final 3 nucleotides form the translation initiating methionine.









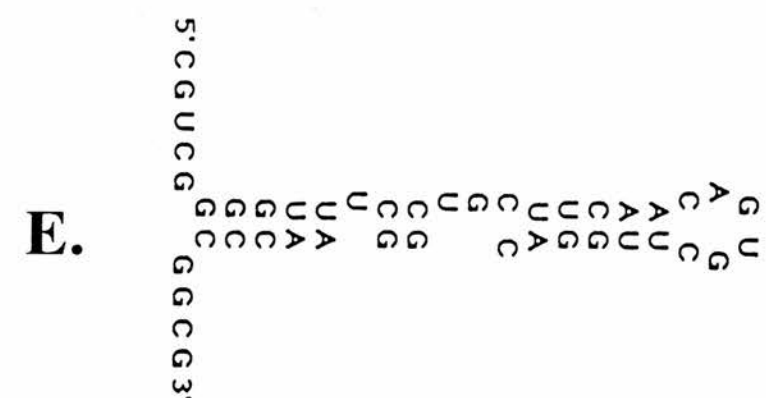


Figure 5.7a (continued).

Figure 5.7b: The nature of the putative NFκβ recognition sequence within the promoter region / 5' UTR of the CYP2E subfamily genes and transcripts. **A:** The NFκβ recognition site general consensus sequence; the totally conserved residues in the sequence are in bold. **B:** Some NFκβ elements located in a variety of genes showing the variation possible within the recognition sites. The totally conserved residues are in bold (Modified from Zabel, et al. , 1991). **C:** The region of the mouse, rat and human CYP2E subfamily genes upstream from the translational initiation codon but downstream from the transcriptional start site, predicted to contain the NFκβ recognition region which is boxed in the figure; all the variations in this element in the CYP2E subfamily have been seen to be present before (**C.**). **D:** The position of the putative NFκβ element within the suboptimally folded 5 ' UTR of the mouse Cyp2e1 cDNA (See Figure 5.7a). Differences within the region containing the putative recognition sequence are shown (**Hu**. human , **Ra.** rat) and the initiation AUG is in hollow type.

A.

5 ' **GGG** ACT TTC **C** 3 '
3 ' **CCC** TGA AAG **G** 5 '

B.

HIV-1	**GGG**ACTTTCC
CMV	**GGG**ACTTTCC
	GGGGATTTCC
SV40	**GGG**ACTTTCC
Ig κ light chain (mouse)	**GGG**ACTTTCC
T cell receptor β	**GGG**AGATTCC
IL-2 receptor α-chain	**GGG**AATCTCC
	GGGAGATTCC
MHC Class II	**GGG**GATTCCC
β-interferon	**GGG**AAATTCC
GM-CSF	**GGG**AACTACC
IL-6	**GGG**ATTTTCC
IL-2	**GGG**ATTTCAC
TNF-α	**GGG**GCTTTCC
Lymphotoxin	**GGG**GCTTCCC
Angiotensinogen	**GGG**ATTTCCC
Serum amyloid-A precursor	**GGG**ACTTTCC
Vimentin	**GGG**GCTTTCC
Interferon regulatory factor-1	**GGG**GAATCCC

C.

```
Human  GTCCTCCCGG GCTGGCAGCA GGGCCCCAGC GGCACCATG
  Rat  ATTCCTACAA GTTTACAGTG GAGCCGCAGT GGCACCATG
Mouse  ATTCCTACAA ATTTAGAGTG GAGCCCCATC GGCACCATG
```

D.

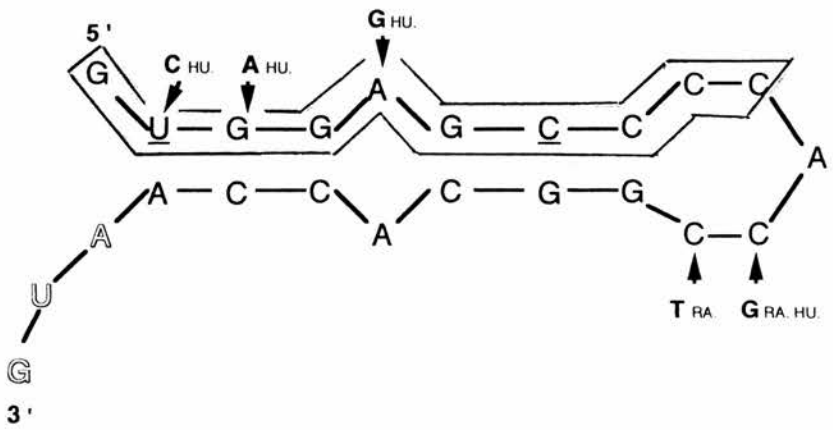


Figure 5.7b (continued).

Section 5.5: The heterologous expression of the Cyp2e1 cDNA

In an attempt to enable the study of some of the chemicals metabolised by Cyp2e1 and its gain an insight into its role in the initiation of carcinogenesis, the Cyp2e1 cDNA was employed in heterologous expression studies. Due to the observed high conservation between the CYP2E genes from several species it would be expected that the biochemical activity of the Cyp2e1 protein would be representative of the actions of CYP2E sufamily proteins in general.

Section 5.5.1: The use of heterologous expression systems in establishing roles for P-450 enzymes in carcinogenesis

The overlapping nature of the substrate profiles metabolised by mammalian P-450, coupled with the differences in the tissue distribution of specific isoforms and the contribution of other xenobiotic metabolising systems, means that an attempt to study the role of a P-450 isoform in the context of the whole organism is complicated. Such a study can be made in situations where sex-differences in P-450 levels occur in a particular organ; in these cases a subtractive approach to the role of a particular P-450 in the metabolism of a certain chemical could be assessed by the ability of a chemical to generate damage in that particular organ in the different sexes. An example of this approach could be argued to have applied in the case of the observed sexual dimorphism of Cyp2e1 in the mouse kidney where the male has higher levels than the female (Chapter 4, Section 4.4). In the mouse kidney, the toxicity of several nitrosamines (Noronha, 1977; Hong *et al.*, 1980; Mohla, 1981; Hawke & Welch, 1985; Ampy & Williams, 1988; Hong *et al.*, 1989) and chloroform (Pohl *et al.*, 1984) produces a much higher injurious effect on the male than the female kidney which may be argued to reflect the sexual dimorphism of Cyp2e1 protein.
One method of determining the potential role of a specific P-450 isoform in the metabolism of a chemical is to employ a reductionist system providing an assay end-point based on genome damage. Applying the knowledge gained from these studies then gives some insight into the possible roles of particular isoforms in the nature of the metabolism and the kinetic parameters of a certain chemical and these observations can then be applied to the observed metabolism of the chemical in the whole animal. Reductionist test-systems have been established employing the genomes of yeast, bacterial and mammalian tissue culture cells as the targets for the activated chemicals (Simula *et al.*, 1993). An example of a widely employed approach to study the mutagenicity of a chemical is the Ame's test where the mutagenic ability of a test chemical is assessed through its ability to generate wild type reversion of a *his*$^-$ strain

of *Salmonella typhimurium* (*S. typhimurium*) (Ames, 1979). Other systems assay other bacterial damage responses such as the expression of components of the SOS operon (Shimada *et al.,* 1989) or the release of bacterial prophages (Elespuru & Yarmdinsy, 1979) both of which activities are elevated in response to DNA damage. The specific roles of particular P-450 isoforms in the activation of the chemical can be assessed by the addition to the assay system of either normal microsome (endoplasmic reticulum enriched fractions) or preparations of microsomes where the activities of certain P-450 isoforms have been induced. More specific roles for certain P-450 isoforms could be determined by using purified P-450 enzymes or inhibitory antibodies to certain P-450 isoforms. These system however have several limitations not least of which is the extracellular nature of the chemical's activation by the activating microsomal or purified enzyme preparations. This short-fall of the test systems was seen to be particularly prevalent in the use of the Ame's test to study the mutagenicity of nitrosamines, the chemicals in carcinogenic terms which would be potentially most relevant to study in connection with the CYP2E subfamily. For example it was seen that neither *N*-nitroso diethylmethylamine (NDMA), or *N*-nitroso diethylethylamine (NDEA), were mutagenic to the *S. typhimurium* test strains, even if activated by the presence of microsomal fractions (McCann *et al.*, 1975; Guttenplan *et al.*, 1976; Yoo *et al.*, 1990; Guengerich *et al.*, 1991). These observation led some researchers to the conclusion that NDMA for example was not mediating its carcinogenic actions directly on the genome (Massour *et al.*, 1983). It is thought that the metabolic activation of nitrosamines involves the initial oxidation of allyl-groups by the action of P-450 yielding alkyldiazohydroxides followed by the generation of diazonium ions which lead to DNA and other macromolecular alkylation (Lai & Arcos, 1981; Shimada *et al.*, 1989; Guengerich & Shimada, 1991, Section 1.7.1) The lack of mutagenic action of nitrosamines, which clearly are carcinogenic *in vivo*, may therefore relate to the extracellular nature of the alkyl-diazonium species generation and their inability to make contact with the bacterial genome.

Attempts have been made to tailor these mutagenicity assays to the testing of nitrosamines. The mutagenic properties of NDMA and NDEA are thought to be enhanced on acetylation of the allyldiazohydroxide species generated from the parent compound. By using *S. typhimurium* tester strains expressing high levels of *o* -acetyltransferase, the enzyme which catalyses this reaction, the mutagenicity of these chemicals have been shown to be elevated (Yamazaki *et al.*, 1992). The mutagenicity of NDMA and NDEA was elevated by the inclusion of microsomes in which the levels of CYP2E1 had been induced, by starvation and acetone treatment, and the mutagenicity was decreased by the inclusion of CYP2E1 inhibitory antibodies; these

observations supported the suggested role for the CYP2E family in the generation of reactive nitrosamine metabolites (Yamazaki *et al.*, 1992).
One clear refinement to allow a better mutagenicity system to be developed for the testing and analysis of the chemicals which CYP2E subfamily proteins could activate to genotoxic metabolites would be the expression of the CYP2E isoform inside the bacterial test strain. Using this approach the problems associated with the extracellular nature of the reactive metabolite generation, the need to generate purified P-450 fractions, or the possibility of cross-reactivity of inhibitory isoform specific antibodies could be overcome as the potential mutagens would be activated within the *S.typhimurium* tester strain itself.

Section 5.5.2: The heterologous expression of proteins in bacterial systems

To express a eucaryotic protein in a bacterial system modifications need to be made to the eucaryotic transcript before it will be translated by a procaryote. Bacterial transcripts contain a conserved sequence just 5' of the initiation methionine codon termed the Shine-Dalgarno (SD), or ribosomal binding (RBS), sequence (Shine & Dalgarno, 1974). This sequence shows a variable degree of complementarity to a region of the ribosomal 16S rRNA and differing rates of translation have been correlated with the degree of complementarity between these two sequences (Atkins, 1979). In general, the SD sequence contains at least four nucleotides from the partial consensus AGGAGG placed between 5 and 9 residues from the initiation codon with the average distance being 7 nucleotides. Eucaryotic messages need to be modified by the addition of an appropriately spaced SD sequence upstream of their initiating codon.
Various other differences between the translation systems employed by pro- and eucaryotic systems have also been noted. For example, differences in codon usage and bias in the messages of these two systems are thought to reduce the translatability of eucaryotic messages in a procaryote. This is seen in the usage of the codon AGA/AGG to encode arginine incorporation in eucaryotic messages. In procaryotic systems the cognate $tRNA_{arg}$ to these codons is not abundant and the ambient levels appear to be associated with the replication rate in *E. coli*.. Thus, eucaryotic messages containing high levels of this codon, taken to be levels above 35% of the arginine codons present in the message, are not expressed well in *E. coli* and interfere with its replication (Garcia *et al.*, 1986; Ayares *et al.*, 1986; Brinkman *et al.*, 1989).
A problem more specific to the expression of eucaryotic P-450 in procaryotic systems is the presence of the N-terminal hydrophobic membrane anchor sequence in the eucaryotic protein absent in their soluble procaryotic counterparts (Section 5.3). Other membrane attached eucaryotic proteins have however been successfully expressed in

procaryotic systems and appear to be incorporated into the bacterial inner membrane (Sarkar *et al.*, 1988; Strittmatter *et al.*, 1988). The soluble *Bacillus megaterium* CYP102, BM-3, protein has been expressed in *E. coli* (Unger *et al.*, 1986; Huiying *et al.*, 1991), and both the bovine 17α-hydroxylase P-450, CYP17, (Barnes *et al.*, 1991) and a fusion between a partial rabbit CYP2E1 cDNA and exon 1 of the rabbit CYP2E1 gene (Larson *et al.*, 1991) have been reported.

Section 5.6: The procaryotic expression systems used to generate Cyp2e1 protein

Two systems were used to generate the heterologous expression of Cyp2e1 in procaryotic cells; the method of operation and use of these two systems is described.

Expression using a modified version of the pKK223-3 (Pharmacia) vector: the parent pKK223-3 vector contains a promoter derived from the fusion of the bacterial *trp* gene promoter and a synthetic DNA fragment containing a consensus -35 region, a partial Pribnow box and a SD (Shine Dalgarno) and *lac I* operator sequence from the bacterial *lac UV5* gene ; collectively this promoter is termed the *tac*II promoter. If the host strain harbouring this plasmid produces the repressor protein encoded by the *lac I* gene the promoter is transcriptionally inactive but can be derepressed by the addition of isopropylthio-β–D-galactosidase (IPTG) which leads to the release of the *lac I* encoded repressor protein from the operator sequence (deBoer *et al.*, 1983). The vector also contains a procaryotic ribosomal terminator sequence (Brosius *et al.*, 1981) and the polycloning site of the pUC8 plasmid.
As a result of the intention to generate expression of the Cyp2e1 protein in *S. typhimurium*, in order to potentially exploit the well characterised mutagenicity tests available in this bacterium, a modified form of the vector was used. The vector requires that the host strain contains an active *lac I* gene and it is recommended that a *lac I*^{q} strain, that is a host with a mutation in the *lac I* gene leading to over-production of the *lac I* encoded repressor, is used in conjunction with this vector. Through the introduction of the *lac I*^{q} gene into the vector itself this requirement is circumvented allowing it to be employed in any host strain. This modification has been performed and the resultant plasmid is termed pKK223-3(mod) (kind gift, Dr I. Hunter, Department of Genetics, Glasgow University).

Expression using the pET system, pET15b: The pET series of vectors place the heterologous transcript under the control of the bacteriophage T7 transcription and translational signals, as well as inserting an appropriately spaced SD sequence upstream of the heterologous message; the T7 DNA dependent RNA polymerase

(T7 "polymerase") is five times as efficient as its cognate *E. coli* protein, allowing the generation of a high level of heterologous transcripts from genes recognised by the T7 polymerase (Studier *et al.*, 1990). The gene encoding the T7 polymerase is placed under the control of an derepressible promoter in the host cell, allowing the expression of the T7 polymerase and so the heterologous transcript to be regulated. In this study the host cell employed was the *E. coli* strain BL21(DE3)pLysE; DE3 encodes a lysogen containing the T7 polymerase under the control of the *lac UV5* promoter and the *lac I* gene whose presence leads to the suppression of the T7 polymerase transcripts by the *lac* operator until it is derepressed by the addition of IPTG. The pET15b vector used in this study has been further refined by the additional inclusion of a *lac* operator sequence downstream of the T7 promoter itself leading to further repression of any heterologous transcripts in the repressed state. The *pLysE* plasmid harboured by the BL21 strain confers chloramphenicol resistance and encodes the T7 lysozyme gene; T7 lysozyme is a bifunctional enzyme which firstly inhibits the small amount of T7 polymerase enzymes which may escape *lac* repression and secondly cuts specific cross-links in the host cell wall allowing easy lysis of the bacteria. The BL21 strain is also mutant in the *lon* and *Omp T* protease genes and so has a reduced ability to degrade both the heterologously expressed protein and the T7 polymerase (Grodberg & Dun, 1988; Studier *et al.*, 1990).

The use of helper phage systems generating integration of the DE3 lysogen allows the potential use of other host strains, and so *S. typhimurium* (Studier *et al.*, 1990).

In the pET15b vector a synthetic oligonucleotide within the polycloning site leads to the addition of six histidine residues fused to the N-terminus of the heterologous protein. The addition of this histidine "tag" facilitates subsequent purification of the heterologously expressed protein through the use of nickel containing resins which selectively bind these histidine residues; the presence of a thrombin recognition site C-terminal to this histidine-tag allows the subsequent release of the native protein (Hochuli *et al.*, 1988; Keyse & Emslie, 1992).

Section 5.6.1: Cyp2e1 expression from the pKK223-3(mod) vector

The full-length Cyp2e1 cDNA was ligated into the *Eco*R I restriction endonuclease sites of pKK223-3(mod); recombinant clones were determined by colony hybridisation using the partial Cyp2e1 cDNA as a probe and the orientation of the clones determined using diagnostic *Bam*H I restriction endonuclease sites. On derepression of the host cells, *E. coli* JM109, this construct was not seen to generate any Cyp2e1 protein as assayed using polyclonal antibodies raised to purified rat CYP2E1 protein. The distance between the vector SD sequence and the Cyp2e1 initiation codon is 22 base-pairs; the

lack of Cyp2e1 protein generation potentially related to the presence of too large a distance between these two elements to allow successful translation of the heterologous transcript.

To generate constructs with a shorter intervening sequences between the SD sequence and the initiation codon, oligonucleotides were generated to the N-, and C-terminals of the Cyp2e1 cDNA. Both the N-, and C-terminal oligonucleotides were designed to contain a *Hind* III restriction endonuclease site and in addition the N-terminal oligonucleotide contained the SD and intervening sequence present in the *E. coli lac Z* gene with the Cyp2e1 initiation codon consequently 7 base-pairs downstream. In addition to the generation of the full-length Cyp2e1 protein two other constructs were engineered in an attempt to make the encoded Cyp2e1 protein more like the procaryotic P-450 proteins. Analysis of the N-terminus of the Cyp2e1 predicted amino acid sequence using a Kyte and Doolittle Hydrophobicity plot programme (Genejockey, Biosoft) shows the presence of an initial stretch of 23 hydrophobic amino acids terminated by three charged residues and then a second stretch of 18 mainly hydorophobic amino acids terminated by a charged residue (Figure 5.8). Alignment comparisons of the N-terminal sequences of all the other CYP2E predicted amino acid sequences shows that these motifs are conserved. In the human CYP2E1 however the lysine and glutamic acid present in the other species as the first and second charged residues are replaced by arginine and lysine, and in the rat and mouse proteins the third positively charged residue (arginine and aspartic acid respectively) is displaced by one amino acid more C-terminal from the analogous histidine residue in the rabbit and human proteins. All these modifications however preserve the overall charge conservation of the N-terminal region of the CYP2E subfamily proteins. The second hydrophobic stretch is terminated by glutamic acid in all the predicted CYP2E subfamily amino acid sequences (Figure 5.5). These observations are in keeping with the proposed insertion of the N-terminus of the P-450 protein, either as a single anchor or a hairpin-like loop, into the endoplasmic reticulum membrane. To generate a Cyp2e1 protein potentially more like the cognate procaryotic soluble P-450 proteins, N-terminus Cyp2e1 deletion mutants, lacking these proposed membrane attachment regions, were generated. Cyp2e1 N-terminal coding region annealing oligonucleotides, still containing the *Hind*III restriction endonuclease site and the *lac Z* SD sequence with an appropriately spaced initiating methionine were generated which would produce N-terminal truncated forms of the Cyp2e1 protein beginning at, after the initiating methionine, trp 30 and leu 48 residues respectively.

These oligonucleotides were used in the polymerase chain reaction (PCR) with the Cyp2e1 cDNA as a template. The PCR products generated were cloned into the *Hind* III restriction endonuclease site of the phagemid vector pTZ18R (Pharmacia) to

allow α-complementation selection of recombinants and generate clonal single-stranded DNA PCR derived cDNA products for sequencing to confirm that the expected regions of the Cyp2e1 cDNA had been amplified. The Cyp2e1 coding sequences and correctly spaced SD sequence were then subcloned into the *Hind* III restriction endonuclease site of pKK223-3(mod), recombinants were screened by colony hybridisation, and the orientation of the cDNA determined by the use of the diagnostic *Bam* H I restriction endonuclease sites.

The pKK223-3(mod) Cyp2e1 truncations, strategy and oligonucleotides employed in the generation of these four pKK223-3(mod) vectors is shown in Figures 5.9 and 5.10 respectively. On IPTG induced derepression of the three constructs with reduced intervening sequences in the *E. coli* JM109 host ccll at 28°C, Cyp2e1, and N-terminally derived truncations of Cyp2e1 were detected after immunoblotting with the polyclonal antibody raised against rat CYP2E1.

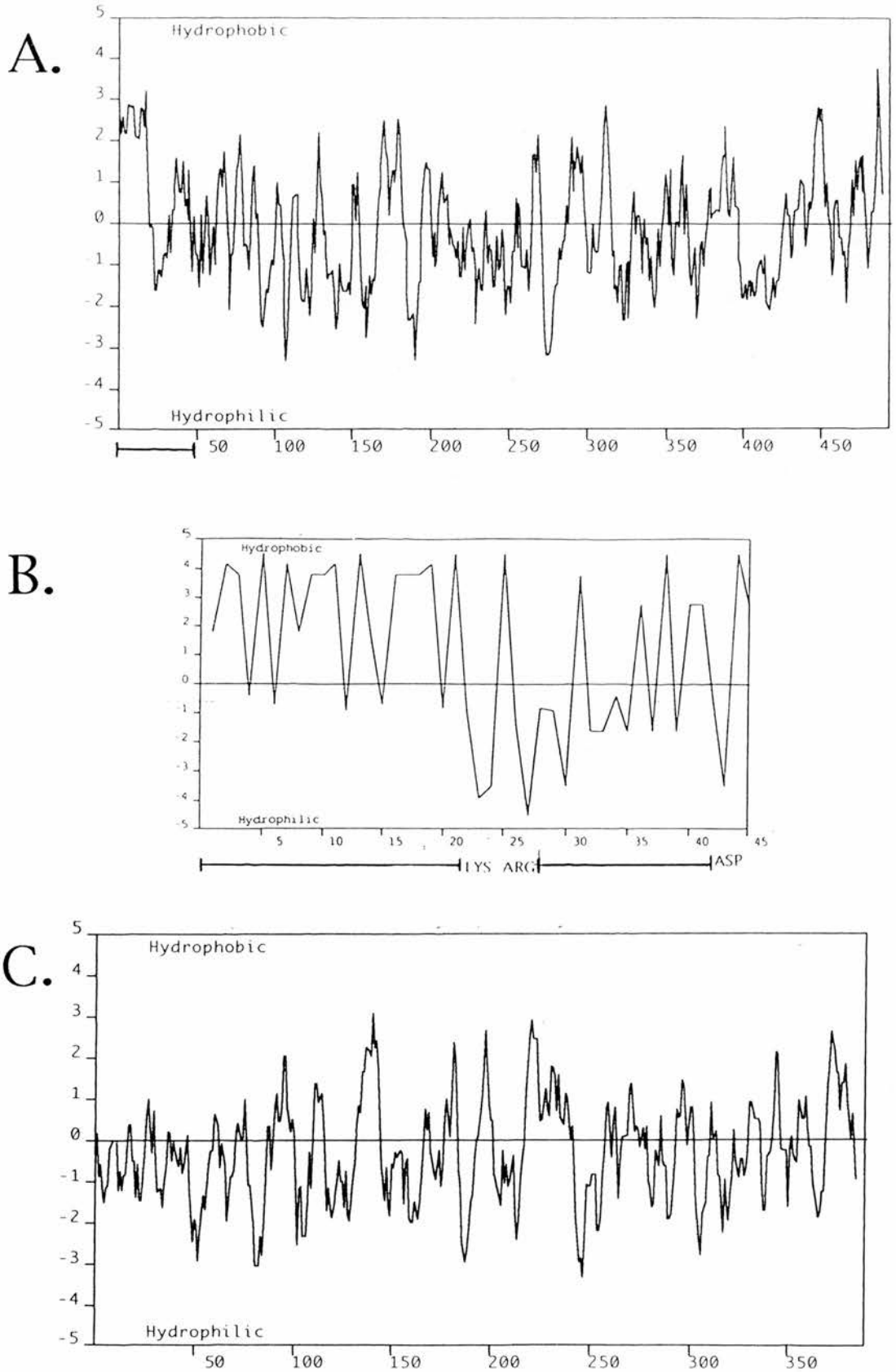


Figure 5.8: Kyte and Doolittle protein hydrophobicity plots of: **A:** Cyp2e1 predicted amino acid sequence. **B:** the first 45 amino acids of the Cyp2e1 sequence representing the portion of the protein predicted to generate the N-terminal membrane anchor. The Lys./Arg. and Asp. stops annotated and the 2 proposed transmembrane segments underlined. The portion of the Cyp2e1 sequence illustrated in B is underlined in A. **C:** CYP101(P450cam) amino acid sequence illustrating the lack of N-terminal hydrophobicity in the soluble procaryotic P-450.

Full-length Cyp2e1:

Hind III Shine-Dalgarno M A V L G I
A ATC TCC CAA GCT TGG G**AG GA**A ACA GCT **ATG** GCG GTT CTT GGC ATC
T V A L A
ACC GTT GCC TTG CTT

Start at Trp 30:

Hind III Shine-Dalgarno M W N L P P
A ATC TCC CAA GCT TGG G**AG GA**A ACA GCT **ATG** TGG AAC CTG CCC CCA
G P F P
GGA CCT TTC CCA

Start at Leu 48:

Hind III Shine-Dalgarno M L K D I P
A ATC TCC CAA GCT TGG G**AG GA**A ACA GCT **ATG** TTG AAG GAT ATC CCC
K S L
AAG TCT TTA

Universal Return C-terminal:

Hind III Stop S R P I V C L V F
AA TCT CCC AAG CTT GGG **TCA** TGA ACG AGG AAT GAC ACA GAT TAC GAA
E R
TTC GCG

Figure 5.9: The oligonucleotides used to generate the full-length and 2, N-terminal truncations of the Cyp2e1 cDNA for expression in *E. coli* by the pKK223-3(mod) vector. The restriction endonuclease sites are annotated; the Shine-Dalgarno sites, termination codon and initiation codons are in bold.

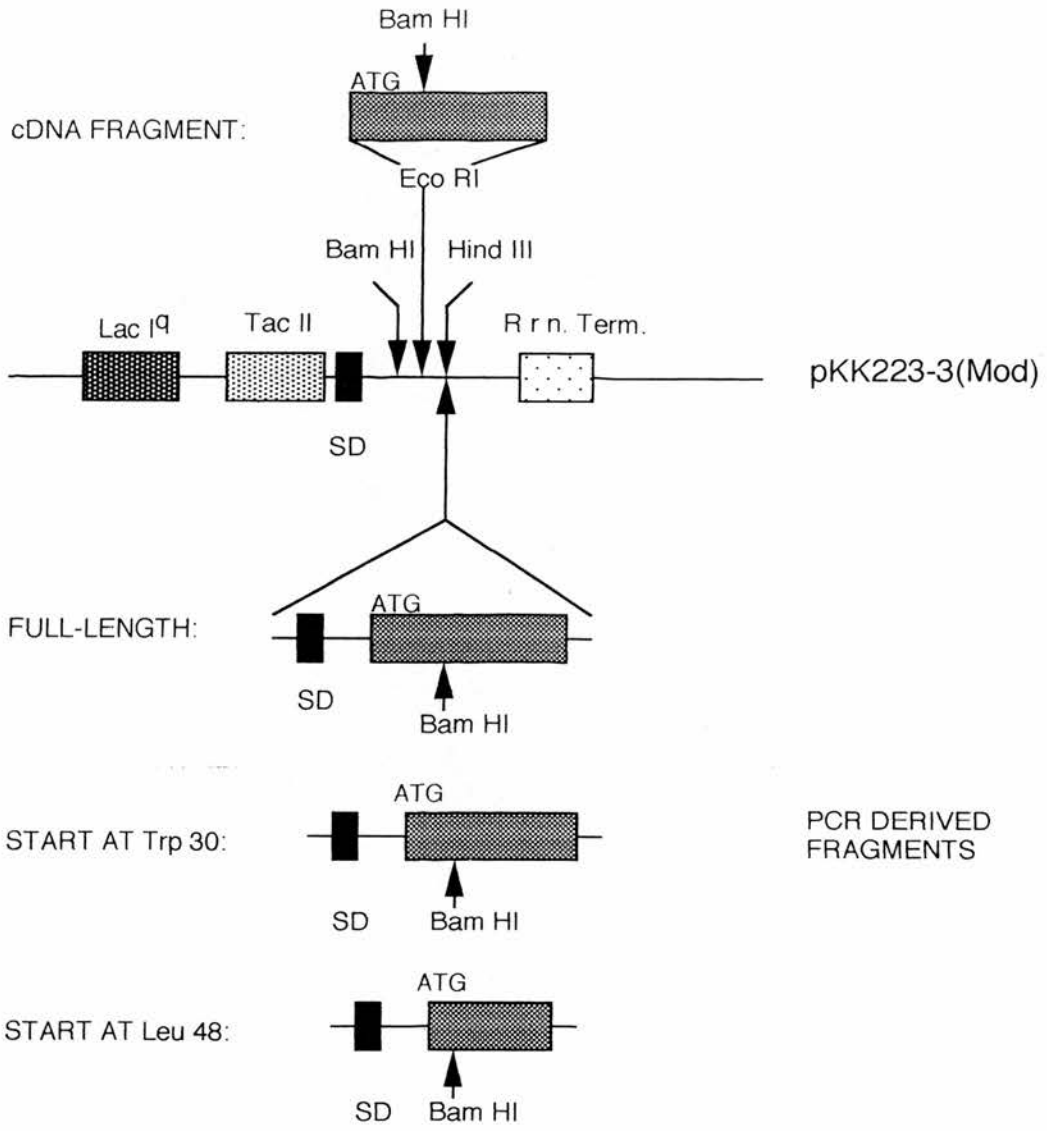


Figure 5.10: The cloning strategies employed to generate the 4 Cyp2e1 pKK223-3(mod) constructs. The initiation (ATG) codon and restriction endonuclease sites in the vector and insert fragments used for cloning are illustrated as are the *Bam* H I sites which allowed the orientation of the insert within the vector. SD: Shine-Dalgarno sequence, Rrn. term: ribosomal terminator sequence. The diagram is not to scale and the pKK223-3(mod) vector is circular.

Section 5.6.2: Cyp2e1 expression using the pET15b vector

To allow the generation of a Cyp2e1 protein direct from the Cyp2e1 cDNA the full-length cDNA was also expressed in the pET15b vector. The full-length Cyp2e1 cDNA was first cloned into the *Eco*R I restriction endonuclease site of the plasmid vector pCITE-2a (Novogen); the recombinant clones were determined by colony hybridisation using the partial Cyp2e1 cDNA as a probe and the orientation of the recombinant clones determined using diagnostic *Bam*H I restriction endonuclease sites. The pCITE-2a plasmid vector has a large polycloning site allowing the subsequent excision of the Cyp2e1 cDNA as an *Nde* I / *Xho* I restriction endonuclease fragment; this fragment was then directionally subcloned into the appropriate restriction endonuclease sites of the pET15b expression vector. This strategy allows the Cyp2e1 coding sequence, its 5' untranslated region and associated restriction endonuclease cloning sites, to remain in frame with the coding sequence of the histidine-tag and thrombin site present in the pET15b vector. The protein generated will therefore contain an additional 32 amino acid residues (M_r 3432). Another Cyp2e1/pET15b construct was generated in which the histidine-tag and thrombin site were removed.The portion of the construct encoding the extra 32 amino acids and the 5' UTR of the Cyp2e1 cDNA was released as an *Nco*I restriction endonuclease fragment and the construct religated, the loss of the *Nco*I restriction endonuclease fragment was confirmed by the loss of a unique *Nde*I restriction endonuclease site contained within the *Nco*I restriction endonuclease fragment .

These manipulations were performed using *E. coli* JM109 as the host strain, the final two constructs were then used to transform the expression *E. coli* host strain BL21 (DE3)pLysE. The BL21(DE3)pLysE host strain is *rec* $^+$, unlike the JM109 strain, and so has a much lower transformation efficiency and hence was not used during the cloning stages of the pET15b/Cyp2e1 constructs. The strategies for construction of these pET15b vectors are shown in Figure 5.11 and the nature of the coding sequences resulting from this strategy are detailed in Figure 5.12.

On derepression of the T7 polymerase by IPTG addition, and subsequent generation of Cyp2e1 transcripts, both these constructs generated immunodetectible Cyp2e1 protein.

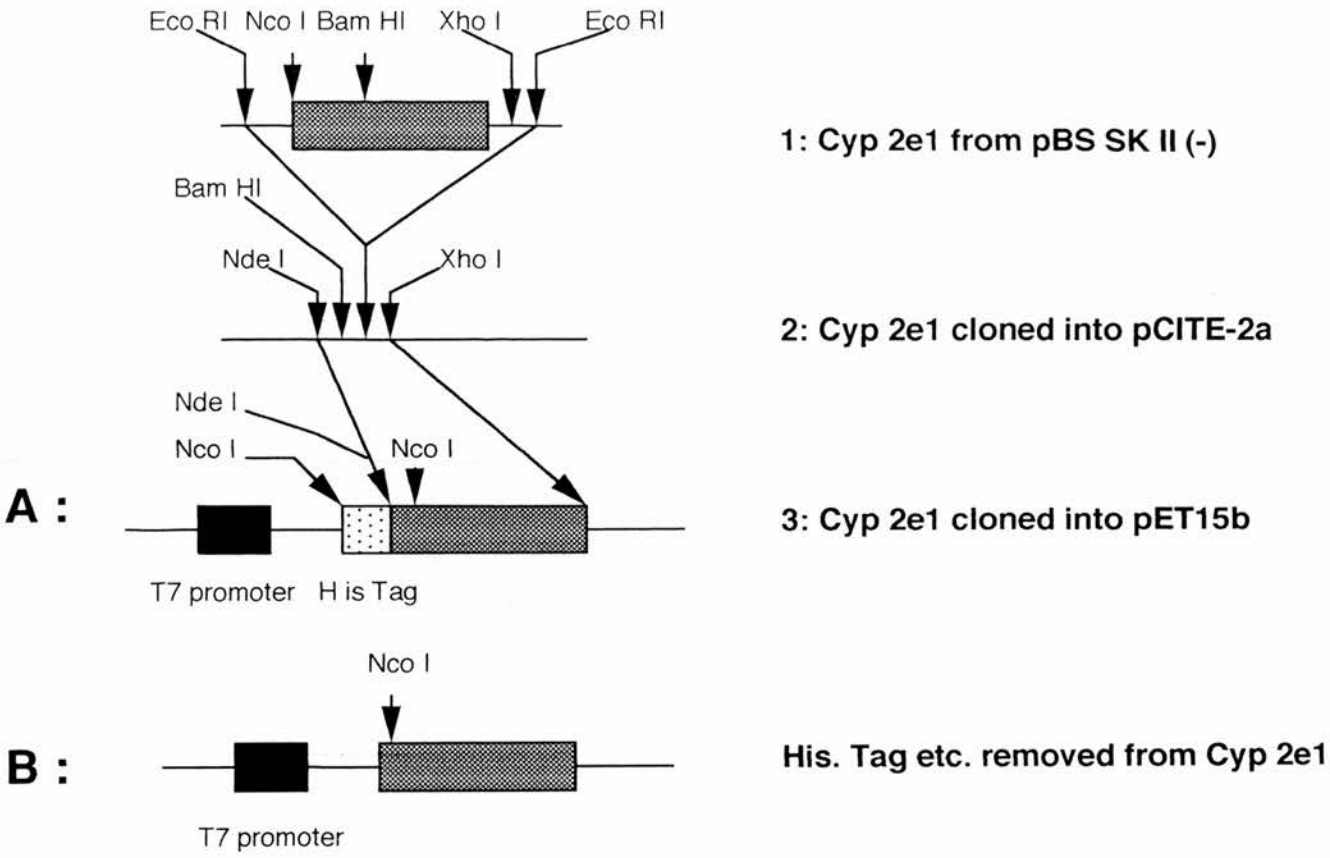


Figure 5.11: The cloning strategy for the generation of the 2, Cyp2e1 constructs in pET15b. Restriction endonuclease sites in the vector and insert fragments used for cloning are illustrated as are the *Bam* H I sites which allowed the orientation of the insert within the pCITE-2a vector. The *Nco* I site corresponds to the initiation methionine of Cyp2e1; the diagram is not to scale and the pET 15b vector is circular.
A: The construct which generated His. Tagged Cyp2e1 protein. **B:** The construct which, following removal of the *Nco* I fragment and religation, removed all non-Cyp2e1 coding regions.

1: *Cyp2e1* cDNA in pBS SK II (-):

Eco RI *Not* I 5' UTR

M A V

GAA TTC CGG GCG GCC GCC ACC ATG GCG GTT .....

Library Linker cDNA .....

2: *Cyp2e1* cDNA cloned into the *Eco* R I site of pCITE-2a:

Nde I *Bam* HI *Eco* RI *Not* I 5' UTR

M A V

CAT ATG GAT ATC GGA TCC GAA TTC CGG GCG GCC GCC ACC ATG GCG GTT

pCITE 2a Library Linker cDNA

3: *Cyp2e1* cloned into the *Nde* I / *Xho* I sites of pET15b:

Nco I His. Tag Thrombin

M G S S H H H H H H S S G L V P

ATG GGC AGC AGC CAT CAT CAT CAT CAT CAT AGC AGC GGC CTG GTG CCG

pET 15b

Site *Nde* I *Bam* HI *Eco* RI *Not* I 5' UTR

R G S H M D I G S E F R A A A T

CGC GGC AGC CAT ATG GAT ATC GGA TCC GAA TTC CGG GCG GCC GCC ACC

pCITE 2a Library Linker

Nco I

M A V

ATG GCG GTT ....

cDNA ....

Figure 5.12: The nature of the coding sequences generated as a result of the cloning strategy employed to generate the Cyp2e1/ pET15b constructs; the strategy progressed chronologically from 1 to 3 (See Figure 5.11). The *Nco* I fragment with an internal diagnostic *Nde* I site which allowed the removal of all non-Cyp2e1 coding sequence from the N-terminal of the protein are illustrated in stage 3.

Section 5.6.3: Subcellular localisation of the Cyp2e1 proteins and N-terminal truncations and relative levels of protein production in the two expression systems

Subcellular fractions from the three pKK223-3/Cyp2e1 and histidine-tagged pET15b constructs were prepared to analyse the localisation of the Cyp2e1 protein. The derepressed cells expressing the various Cyp2e1 constructs were harvested by centrifugation, resuspended in a sucrose buffer and then disrupted by passage through a French Press; enriched membrane fractions were prepared using a modification of the method of Yoshida and Aoyoma (1984). The cell debris, consisting of whole cells and large cellular components such as the cell walls, were removed by a 5,000g centrifugation and a membrane enriched fraction was then sedimented from the resultant supernatant by two periods of ultracentrifugation at 100,000g and resuspension in sucrose buffer to remove associated cytosolic proteins. The two crude fractions were then assessed for the presence of the Cyp2e1 protein by immunoblotting using the polyclonal antibody raised against purified rat CYP2E1 protein. The results from these studies are shown in Figure 5.13.

The full-length protein generated in pKK223-3(mod) appeared to be directed predominantly to the membrane enriched fraction with a relatively small amount being present in the cellular debris. In contrast, the two truncated Cyp2e1 forms generated from this vector locate proportionately more in the cell debris with a smaller proportion being associated with the membrane enriched fraction. The truncated Cyp2e1 protein forms appear to have been attacked by host cell proteolytic degradation systems, products of which are seen in the cell debris fraction. The observation that the truncated forms of the Cyp2e1 protein still associate with the membrane enriched fraction, and may therefore still be attached to the membrane, is intriguing. The currently accepted model for eucaryotic P-450 membrane insertion suggests that a helical-hairpin, generated by the two hydrophobic stretches removed in the Cyp2e1 truncations, pass through the membrane holding the rest of the enzyme within the cytosol; this membrane attachment region is postulated to represent the only divergence between a common protein structure adopted by the eucaryotic P-450 and their soluble procaryotic counterparts (Nelson & Strobel, 1988). A similar result was seen in the expression of a rabbit CYP2E1 partial cDNA and exon 1 gene fusion; an N-terminal truncation initiating at amino acid 29, the equivalent of the removal of the first predicted membrane span in the pKK223-3(mod) trp 30 Cyp2e1 construct, resulted in a protein which was still membrane associated (Larson *et al.*, 1991). Membrane attachment may still be mediated by the second hydrophobic amino acid stretch in these constructs, this however is not a possibility in the pKK223-3(mod) leu 48 Cyp2e1 truncation construct in which both the predicted membrane spanning regions have been removed. Other

models for P-450 membrane association have postulated the existence of up to 10 trans-membrane helices (Hudecek & Anzenbacher, 1988); the observed membrane association of the Cyp2e1 truncation without either of the proposed membrane spanning regions may however not be the result of membrane spanning regions *per se* but the effect of strong membrane associations with some of the regions of hydrophobicity spread along the length of the protein (Figure 5.8). It would be predicted that, for energetic reasons, these hydrophobic regions of the protein would be held within the interstices of the P-450 molecule but some may be present on the surface of the structures and so interact with the membrane.

One problem commonly encountered in the heterologous expression of proteins in procaryotic systems is that of "inclusion body" formation. Inclusion bodies are formed from aggregates of insoluble heterologous protein, and several host proteins such as ribosomal and RNA polymerase subunits, which become sequestered in the bacterial cytoplasm. The processes leading to the formation of inclusion bodies are unclear and may not solely relate to the level of heterologous protein produced although this factor does play a clear role; the proteins within the inclusion body are effectively extracellular and as such are not acted upon by the degradational systems within the cell (Schein, 1989; Shoemacker *et al.*, 1989). When the morphology of the host cells expressing Cyp2e1 protein was viewed using light microscopy, it appeared the same as that observed in cells not containing the pKK223-3(mod) constructs. The lack of a filamentous appearance or the presence of refractile granules in the *E. coli* cells expressing Cyp2e1 protein suggests that the constructs have not generated inclusion bodies. The fact that the truncated Cyp2e1 forms are being degraded by the host proteolytic systems also suggests that they have not become precipitated into inclusion bodies as this event would have made them resistant to proteolytic attack.

The different pKK223-3 Cyp2e1 constructs generated different levels of Cyp2e1 protein; the progressive N-terminal truncation of the Cyp2e1 cDNA leads to an increase in the levels of Cyp2e1 protein produced even though identical promoter and associated sequences are present in each instance. This observation may be accounted for by the context of the codon encoding the first methionine; sequences which generate an unstructured environment surrounding the initiation codon, such as a poly-adenosine region, lead to higher levels of heterologous protein translation (Balbas & Boliva, 1990). The codons adjacent to the initiating methionine have also been seen to affect the levels of resulting protein translation; it was seen that the presence of adenosine and uridine residues at positions 4 and 5 (Stormo *et al.*, 1982), and similarly GCT (alanine) as the second codon (Looman *et al.*, 1987) increased the level of protein expression . The first six codons of the three Cyp2e1 pKK223-3 constructs are shown in Figure 5.9; the most truncated -49 amino acid construct generates a message

containing the highest uridine and adenosine content, and with 50% adenosine in codons 4 and 5 suggesting that these features may relate to the higher protein levels produced. No protein expression was produced from the native bovine CYP17 cDNA until such modifications were introduced to these regions of the transcript (Barnes *et al.*, 1991). In contrast to the suggested effect of alanine as the second codon increasing the level of expression of a protein, the full-length construct is the only Cyp2e1 message, with the lowest level of expression, which contains alanine in codon two (Figure 5.9). These two observations highlight the problems associated in attempting to make generalisations in the factors in a transcript which affect the level of protein that will be expressed.

The majority of the Cyp2e1 protein generated from the pET15b vector was seen to be present in the cell debris and relatively little associated with the membrane enriched fraction (Figure 5.13). The level of full-length protein generated from the T7 promoter is higher than that from the pKK223-3(mod) construct due to the higher efficiency of the T7 polymerase. Removal of the 32 amino acid leader in the second *Nco* I cut pET15b construct did not affect either the level, or cellular localisation of the Cyp2e1 protein produced. The cellular localisation and observed lack of degradation, in association with a differing morphology in the pET15b Cyp2e1 expressing host cells when viewed under light-microscopy, suggests that the majority of Cyp2e1 protein produced by this vector is present in inclusion bodies. The use of the pET15b Cyp2e1 expression vector constructs in the study of the mutagenicity of Cyp2e1 would therefore be negligible. The histidine-tagged construct could allow however the rapid single step purification of the Cyp2e1 protein through the use of a nickel agarose affinity column (Keyse & Emslie, 1992); this may allow the production of an antibody specific to the mouse Cyp2e1 protein removing the problem of cross-reactivity present when the rat CYP2E1 polyclonal antibody is used in some tissues in the mouse (Figure 4.3 and 4.4).

The pKK223-3(mod)Cyp2e1 constructs could potentially be used therefore in mutagenicity testing to be carried out in *S. typhimurium.* Although some success has been obtained in regenerating active proteins from inclusion bodies (Marston, 1986; Sammuelsson *et al.*, 1991) this approach would not be relevant in the case of the pET constructs, with their production of Cyp2e1 inclusion bodies, as this would not allow the direct use active intracellular Cyp2e1 protein in mutagenicity testing.

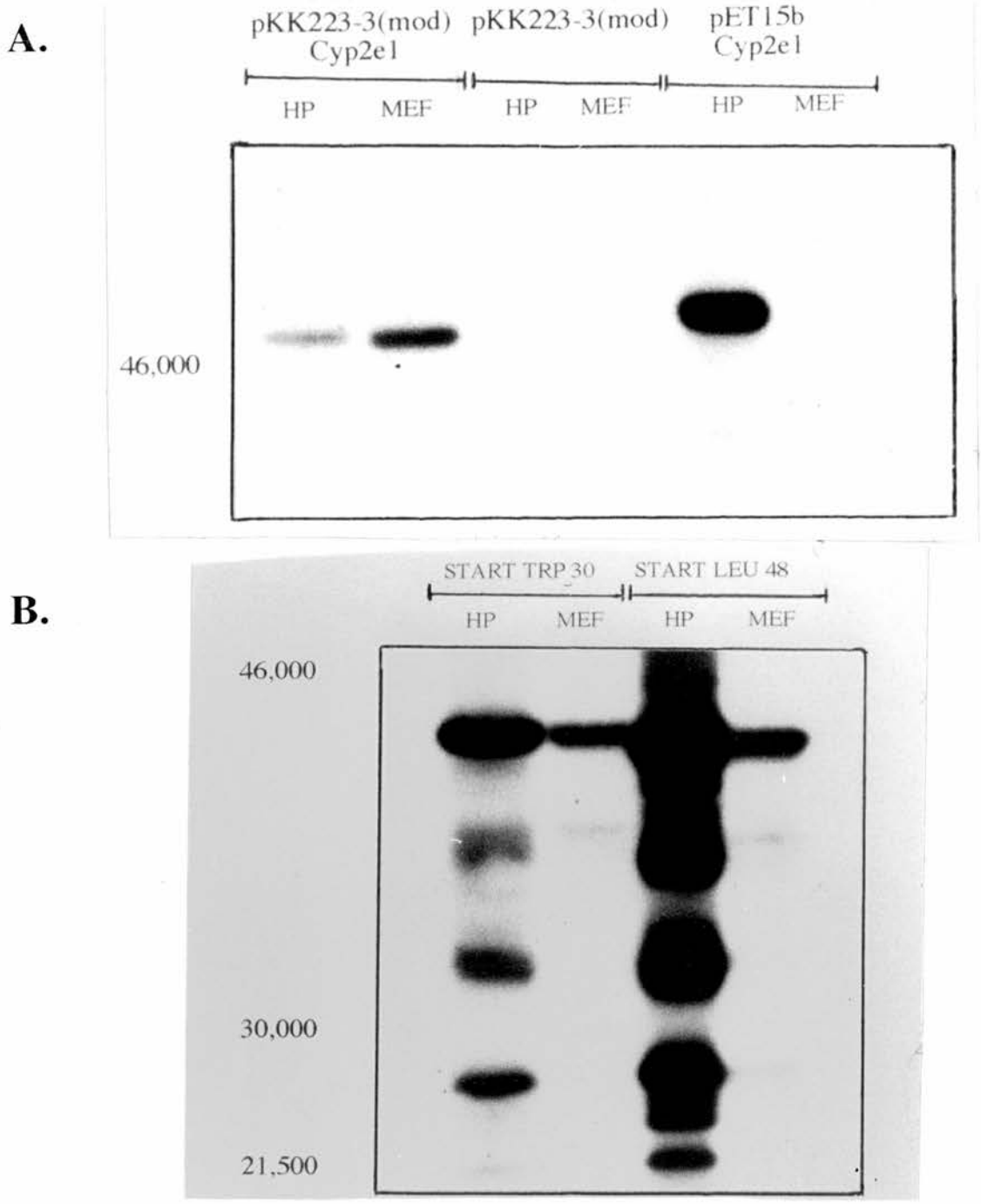


Figure 5.13: The subcellular localisation of the Cyp2e1 protein expressed in *E. coli* determined using the polyclonal antibody raised to purified rat CYP2E1 protein. **HP:** "Heavy pellet", the protein fraction containing cell walls, whole cells and insoluble material isolated following low speed centrifugation of French pressed samples. **MEF:** Membrane enriched fraction, isolated after two ultracentrifugation steps of the supernatant resulting from the initial low spped centrifugation. 10μg of protein was loaded in each track. **A:** From left to right: Full-length Cyp2e1 generated from pKK223-3(mod), control *E. coli* protein from cells transformed with pKK223-3(mod) alone and pET15b/Cyp2e1+ His.Tag transformed cells. **B:** The 2 N-terminal truncations of Cyp2e1 in pKK223-3(mod).

Section 5.6.4: Transformation of *S. typhimurium* strains with the pKK223-3(mod) Cyp2e1 constructs

The pKK223-3(mod) Cyp2e1 constructs were used to transform the *S. typhimurium* strains TA1538 and LR5000 by electroporation. The constructs were also used to transform the BL21(DE3)pLysE *E. coli* strain to investigate if higher levels of expression could be produced in *E. coli* strains other than JM109. The host cells were lysed by sonication and the level of Cyp2e1 protein generated assayed by immunoblotting of equal protein concentrations of total host cell protein (Figure 5.14). It is clear that the pKK223-3(mod) constructs will generate Cyp2e1 protein in *S. typhimurium* with the highest levels being produced by the LR5000 strain; the BL21(DE3)pLysE *E.coli* strain produced relatively less Cyp2e1 protein than the JM109 strain. These observations also applied to the truncated versions of the pKK223-3(mod) Cyp2e1 constructs.

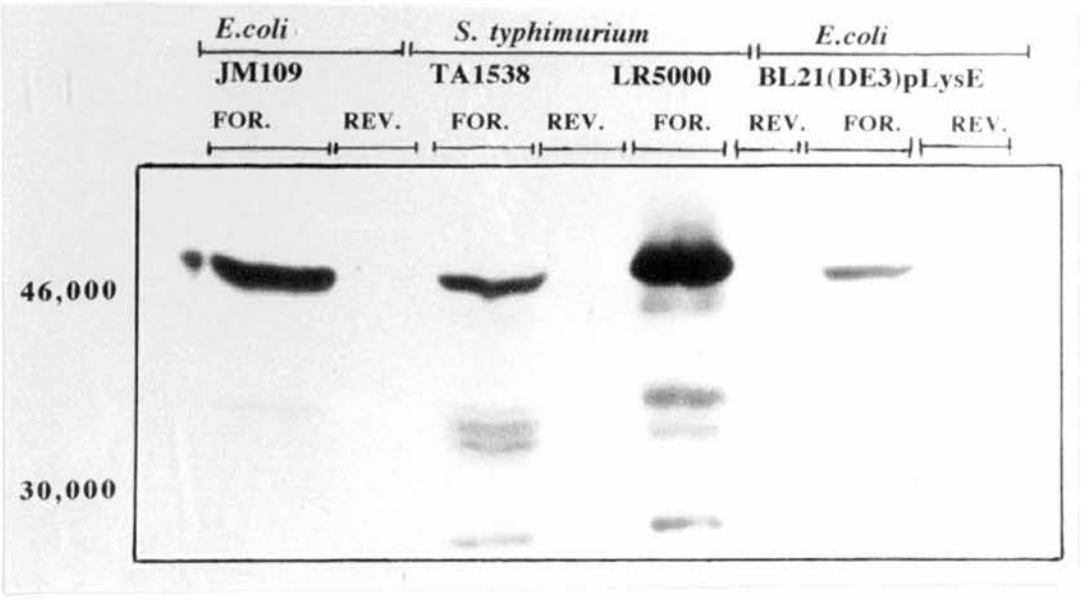


Figure 5.14: The detection of full-length Cyp2e1 protein produced from the pKK223-3(mod) Cyp2e1 construct in *S. typhimurium* and *E. coli* determined using polyclonal antibodies raised to purified rat CYP2E1 protein. The bacteria, as stated, were transformed with either the pKK223-3(mod) Cyp2e1 construct in the correct, **FOR.**, or reverse, **REV.** , orientation for expression of the Cyp2e1 protein. The cells were disrupted by sonication and 10μg of total cellular protein was loaded per track.

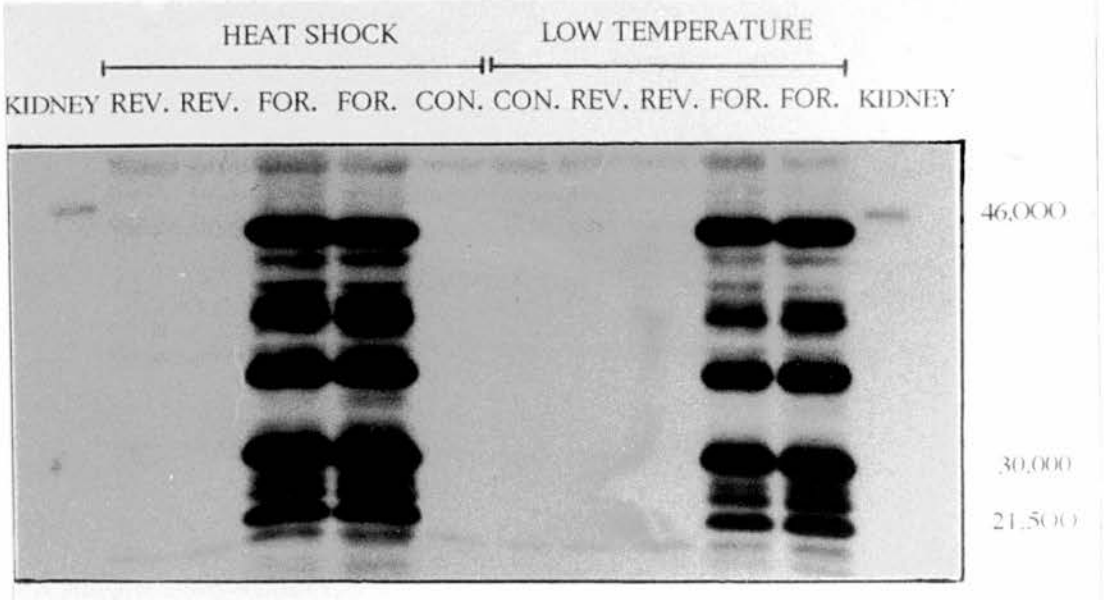


Figure 5.17: The detection of N-terminally truncated Cyp2e1 protein produced from the pKK223-3(mod) Trp 30 Cyp2e1 construct in *E. coli.* using polyclonal antibodies raised to purified rat CYP2E1 protein. **Heat shock:** The cells were heated at 42°C prior to derepression of the *tac II* promoter. **Low temperature:** The cells were incubated at 20°C following derepression of the *tac II* promoter. The bacteria, were transformed with either the construct in the correct, **FOR.**, or reverse, **REV.** , orientation for expression of the Cyp2e1 protein. The cells were disrupted by sonication and 10μg of total cellular protein was loaded per track.

Section 5.6.5: Spectral and metabolic activity of the heterologously expressed Cyp2e1 proteins

The presence of a haem group within a P-450 holoenzyme can be detected on analysis of the "reduced-difference" spectra. The reduction of the haem group and the analysis of the absorbence spectra produced on binding of carbon monoxide to the haem relative to non-carbon monoxide bound haem leads to an absorbence peak at 450 nm if haem is present (Omura & Sato, 1962).

In the heterologous expression of bovine CYP17 in *E. coli* it was demonstrated that a reduced difference spectra could be produced from whole host cells expressing mammalian P-450 protein (Barnes *et al.*, 1991). This approach was repeated using whole cell expressing Cyp2e1 from the pKK223-3(mod) Cyp2e1 constructs in both *E. coli* and *S. typhimurium* strains and the pET15b Cyp2e1 expressing *E. coli* strain constructs, but no peak could be detected at 450 nm. A large absorbence trough was detected proceeding from 460 nm and extending to around 430 nm where a peak rose at approximately 415 nm. These spectral features were observed in *S. typhimurium* and *E. coli* cells whether they were expressing Cyp2e1 protein or not (Figure 5.15). Analysis of the cell debris and membrane enriched fractions of all the cells expressing constructs did not reveal a 450 nm peak and no difference in the spectra produced by the expressing and non-expressing hosts was obtained. This spectral pattern detected in the cells expressing Cyp2e1 was observed in the examination of the cell membranes of *E. coli* expressing a rabbit CYP2E1 cDNA and gene fusion (Larson *et al.*, 1991); it is not clear why it was not recorded in the spectra obtained from whole *E. coli* cells expressing bovine CYP17 protein (Barnes *et al.*, 1991).

The spectral patterns seen in the cell preparations are diagnostic of host endogenous cytochromes; the patterns produced suggest the presence of a membrane bound cytochrome oxidase, or cytochrome-o, which generates a peak between 410-419 nm and a trough between 560-547 nm in variety of bacteria studied (Chance, 1961; Revsin & Brodie, 1969; Miller & Gennis, 1983). In common with the P-450 cytochromes, cytochrome oxidase contains a protohaem prosthetic group; both cytochrome groups are therefore classed as b-type cytochromes (Lemberg & Barret, 1973). The study expressing rabbit CYP2E1 protein also noted an absorbence minium at 444 nm within the area of the cytochrome oxidase trough indicative of bacterial cytochrome-d; this cytochrome contains a dihydroporphyrin prosthetic group and is expressed when oxygen becomes limiting during log or stationary phases of growth (Kita *et al.*, 1984a, Kita *et al.*, 1984b), the cultures used for the Cyp2e1 expression were well aerated and so did not express this cytochrome.

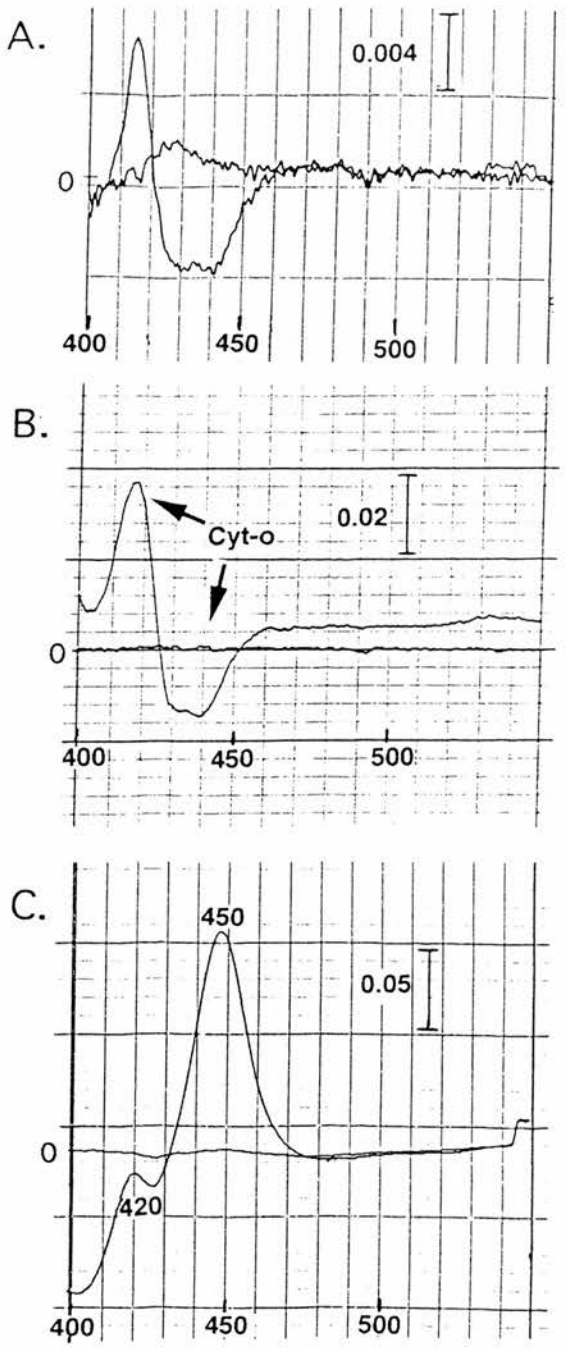


Figure 5.15: Analysis of the heterologously expressed Cyp2e1 protein for haem content by reduced difference spectroscopy. **A:** The reduced difference spectra produced by whole *E. coli* expressing Cyp2e1. **B:** The reduced difference spectra produced by the membrane enriched fraction of *E. coli* expressing Cyp2e1 (2mg/ml). The trough proceeding from 460nm and terminating in a peak at 415nm is characteristic of cytochrome-o (**Cyt.-o**). **C:** The reduced difference spectra produced by rat liver microsomes (2mg/ml) demonstrating the characteristic 450nm and 420nm peaks of haem containing P-450 holoenzyme.

The fractions prepared from the expressing strains were tested for their ability to hydroxylate *p*-nitrophenol, a substrate for the CYP2E subfamily (Reinke & Moyer, 1985; Koop *et al.*, 1989). The supernatant obtained from the initial 5,000g centrifugation step in fraction preparation was seen to provide reducing equivalents to the bovine CYP17 protein expressed in *E. coli* when added at a ratio of 1:1 by protein concentration to the assay mixture (Barnes *et al.*, 1991) and this fraction was added to the *p*-nitrophenol assay mixture. None of the fractions produced any detectable *p*-nitrophenol hydroxylation, suggesting that the Cyp2e1 protein produced is not metabolically active.

The spectral characteristics of the heterologous Cyp2e1 protein generated suggest that, although the apoprotein can be detected immunologically, the protein produced is not incorporating a protohaem iron prosthetic group. Poor haem incorporation into P-450 apoprotein has been a common phenomenon in yeast (Oeda *et al.*, 1985; Murakami *et al.*, 1986; Murakami *et al.*, 1987; Fujita *et al.*, 1990) and bacculoviral (Asseffa *et al.*, 1989) expression systems.

Section 5.6.6: Increasing the protohaem incorporation in the Cyp2e1 protein

It is thought that the incorporation of the protohaem moiety into the apoprotein is not enzymatically catalysed (Tokunga & Sano, 1966). The protohaem prosthetic group appears to be lost and regained by the P-450 apoprotein as demonstrated by the relative half lifes of the protohaem (short) and apoprotein (long) moieties in pulse-chase studies (Section 4.7.1). This suggests that the loss of the protohaem does not lead to the complete deformation of the apoprotein structure allowing its reincorporation. It may however be the case that the initial generation of the P-450 structure needs the presence of the protohaem, with its contribution of a large hydrophobic surface within the molecule, in order to fold correctly. If this is the case initial protohaem incorporation in the P-450 apoprotein molecule may be the key to generating a correctly folded molecule capable of protohaem incorporation and exchange.

In an attempt to increase initial haem incorporation into the Cyp2e1 apoprotein the levels of protohaem within the host cells were elevated. Protohaem is generated through a series of steps in bacteria from glycine and succinyl-CoA precursors and the basic pathway for its generation is thought to be the same in all organisms (Barret, 1969). In a bacculoviral expression system the level of protohaem incorporation into heterologous P-450 apoprotein could be increased by the addition of hemin although this predominantly produced an absorbence spectra at 420 nm suggested to be indicative of a loss of P-450 structure (Asseffa *et al.*, 1990). This could not be used in the case of

bacterial expression however as both *S. typhimurium* and *E. coli* are not capable of taking up extracellular hemin (Sarsaman *et al.*, 1970).

Studies on *S. typhimurium* (Sarsaman *et al.*, 1970) and *E.coli* (Wulf, 1964) mutants deficient in the generation of haem prosthetic groups showed that cytochromes could be produced following addition of the porphobilogen precursor δ-aminolaevulinic acid (δALA). The production of δALA is suggested to be the key regulatory step in the production of protohaem and may be controlled by the ambient protohaem levels in a cell (Burnham & Lascelles, 1963).

δALA was added to the bacterial cultures at a concentration of 6 x 10^{-6} M, the concentration previously used by Wulf (1964), prior to the derepression of the Cyp2e1 constructs and membranc enriched fractions were prepared. Addition of δALA to the cultures produced a profound effect on the levels of the endogenous bacterial cytochromes; the membrane enriched fractions were a deep red compared to the control cultures paler appearance and an increased absorbence trough and peak were detected. However, a peak at 450 nm could still not be detected in the difference spectra produced (Figure 5.16). The bacterial cytochrome-o , in common with P-450, contains a protohaem prosthetic group. This demonstrated that addition of δALA to the bacterial culture medium leads to increased protohaem incorporation into the endogenous cytochrome-o, but not the heterologously expressed Cyp2e1 protein. This suggests that the lack of protohaem incorporation into the Cyp2e1 protein relates to an inability of the hosts to correctly insert the protohaem group rather than to a lack of protohaem groups available *per se* . If this is the case then the lack of protohaem in the Cyp2e1 apoprotein may relate to the nature of the heterologous protein folding in the host cell and this possibility was investigated.

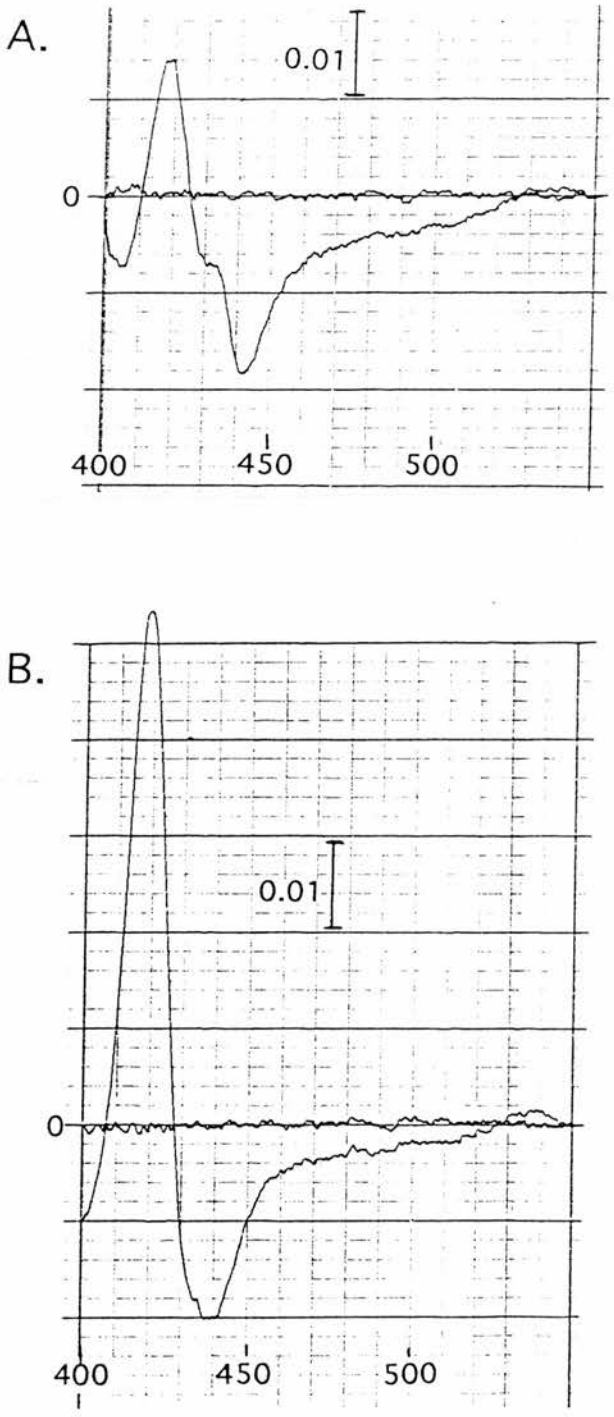


Figure 5.16: Analysis of the effect on the reduced difference spectra of the addition of δ-aminolaevulinic acid (δALA) to the grown media of *E. coli* expressing Cyp2e1 protein. **A:** The reduced difference spectra produced by the membrane enriched fraction (1mg/ml) of *E. coli* expressing Cyp2e1 in the absence of δALA in the media. **B:** The reduced difference spectra produced by the membrane enriched fraction (1mg/ml) of *E. coli* expressing Cyp2e1 with δALA in the media; the massive increase in the peak at 415nm may be indicative of an elevation of cytochrome-o content in the samples.

Section 5.6.7: Cyp2e1 folding and protohaem incorporation

The lack of protohaem incorporation into the Cyp2e1 protein may reflect the lack of presentation of a structure into which the prosthetic group can become correctly inserted; the completion of the P-450 folding may then follow the prosthetic group insertion.

The processes leading to the folding of a protein are not clearly understood and are thought to be mainly driven by the energetic driving force of hydrophobic portions of the protein coalescing to escape the ionic cytoplasmic environment with the secondary structural motifs then formed by a shuffling of possible structural permutations within this complex until the most stable form is generated. This process is complicated by prosthetic group addition which may sterically limit the degree of such structural permutations and this may be the case in the generation of the P-450 protein structure (Gething & Sambrook, 1992).

Although many denatured proteins can be successfully refolded *in vitro*, suggesting that the information for correct folding is present within the primary sequence of the polypeptide itself, other controls impinge upon and further complicate folding; an example of the factors associated with protein folding within a cell are the molecular "chaperone" proteins (Georgolopoulos, 1992; Gething & Sambrook, 1992). Within *E. coli* the chaperone proteins DnaK and GroEL, which are thought to be the equivalent of the mammalian heat-shock proteins hsp70 and hsp60, interact with the nascent protein as it is generated by the ribosome and promote its correct folding. These proteins are thought to exert their action possibly by holding the nascent protein within a hydrophobic environment and preventing the aggregation and precipitation of unfolded proteins (Beckmann *et al.*, 1990). Chaperones are also thought to recognise particularly hydrophobic portions of a protein, such as membrane insertion or secretion signals, and bind them to prevent their incorporation into the rest of the protein structure (Randall & Hardy, 1986; Chiroco *et al.*, 1986). Chaperone levels are seen to be induced in cells with an increased amount of incorrectly folded protein for example following a heat-shock, potentially to facilitate the correct refolding of the proteins (Parsell & Sauer, 1989).

If the Cyp2e1 apoprotein is being incorrectly folded some diagnostic features may be expected in the resultant protein generated. In procaryotic systems it is suggested that proteolytic attack is initiated principally on proteins present in an unfolded, or incorrectly folded, state and studies have shown that the introduction of amino acid mutations within a protein which inhibit its correct folding lead to increased proteolysis (Parsell & Sauer, 1989; Parsell & Sauer 1990; Parsell *et al.*, 1989). Other features of a protein, such the nature of the N-, and C-termini, have also been associated with

stability in bacteria (Bachmair *et al.*, 1986). Previously it had been suggested that *E.coli* mutants lacking DnaK were deficient in proteolysis (Strauss *et al.*, 1988) but now it is apparent that the lack of chaperone molecules leads to the generation of insoluble protein aggregates, as they are unable to fold correctly, leading to inclusion body formation and resistance to proteolysis (Parsell & Sauer, 1989). Particularly in the case of the truncated forms of the pKK223-3(mod) generated Cyp2e1 proteins it is clear that the expressed protein is subject to proteolytic attack and so possibly incorrectly folded. The relative resistance of the full-length construct to proteolysis, in the absence of morphological changes associated with inclusion body formation, suggests that the presence of the membrane insertion signal may encourage binding by chaperones facilitating its correct folding. This suggestion does not apply to the pET15b Cyp2e1 constructs where the protein formed inclusion bodies and so is resistant to proteolytic attack (Figure 5.13).

In an attempt to increase the level of correctly folded Cyp2e1 molecules and so the possibility of protohaem incorporation, chaperone proteins were induced within the host cells by a period of heat-shock at 42 °C (Parsell & Sauer, 1990a) prior to derepression of the Cyp2e1 expression constructs. In an additional experiment by culturing the expressing cells at 20 °C it was hoped that by slowing the folding of the heterologous Cyp2e1 protein the generation of correct folding and protohaem incorporation may be encouraged. Neither of these approaches however generated any increase in the level of protohaem incorporation measured by difference spectra, or appeared to generate a more correctly folded apoprotein structure as assayed by the level of proteolysis acting upon the truncated Cyp2e1 constructs (Figure 5.17).

Section 5.6.8: Why does the rabbit CYP2E1 apoprotein, unlike the mouse Cyp2e1 protein, incorporate protohaem upon heterologous expression ?

It is not clear why rabbit CYP2E1 and mouse Cyp2e1, two highly similar P-450 proteins possessing 79% amino acid identity (Table 5.1), should behave so differently in virtually identical heterologous expression systems (Table 5.4). One possibility is that a very fine balance exists as a result of the levels of protein produced by the expression vector between a swamping of the ability of the host cell to fold the protein correctly and allowing enough protein to be produced to enable detectable levels of P-450 holoenzyme to be generated. The extremes of this scale may be predicted to be inclusion body formation as the result of excess heterologous protein generation (as noted in the use of the pET15b Cyp2e1 constructs) and undetectable haem levels as the result of insufficient heterologous protein levels (as seen in the case of the pKK223-3(mod) Cyp2e1 constructs. It may be the case therefore that the rabbit CYP2E1 cDNA,

in common with the bovine CYP17 expression system, as a result potentially of structural differences spread across the protein, has satisfied the requirements of this fine balance and is producing protein at a rate and level to allow correct folding and protohaem incorporation to be detected.

Table 5.4: The vectors employed and conditions which have generated the successful expression of mammalian P-450 proteins in procaryotic systems to-date compared with the conditions used with the pKK233-3(mod) constructs in this study:

P-450 produced	Vector	Promoter	*E.coli*	Growth	Derepression	Ref.
Rabbit CYP2E1	pKK3a (pKK223-3 derived by addition of pUC origin of replication to increase copy number).	*tac* II	JM105	37°C	2mM IPTG	Larson, *et al.* 1991. Porter, *et al.* 1991.
Bovine CYP17	pCWOri$^+$ (contains the *lac* I^q gene to allow expression in any host).	Two copies of the *tac* promoter	JM109	28°C	1mM IPTG	Barnes, *et al.* 1991.
Mouse Cyp2e1	pKK223-3(mod)	*tac* II	JM109	28°C	1mM IPTG	

The lack of protohaem incorporation into heterologously produced P-450 proteins has hindered both yeast (Oeda *et al.*, 1985; Murakami *et al.*, 1986; Murakami *et al.*, 1987; Fujita *et al.*, 1990) and bacculoviral (Asseffa *et al.*, 1989) heterologous expression systems. Low levels of protohaem incorporation clearly also occur in the use of procaryotic expression systems to study P-450 activity and this phenomena has been observed in connection with other P-450 isoforms expressed in similar procaryotic systems in this laboratory. Some workers have stated that mammalian P-450 can not be functionally expressed in procaryotic systems (Cullin & Pompon, 1988) although the possibility of generating functional mammalian P-450 in procaryotic systems has now been demonstrated (Barnes *et al.*, 1991; Larson *et al.*, 1991). The paucity of data relating to the use of procaryotic expression systems to study mammalian P-450 activities, given its extremely tantalising potential in relation to mutagenicity testing, suggests that the production of inactive apoprotein P-450 unable to incorporate protohaem may not be an uncommon phenomenon. The production of functional P-450 protein may require that a unique set of experimental conditions may need to be correctly generated.

Section 5.7: The expression of Cyp2e1 in *Saccharomyces cerevisiae*

Although it is feasible to produce active mammalian P-450 in procaryotic heterologous expression systems (Barnes *et al.*, 1991; Larson *et al.*, 1991), it is possible that a fine balance between the protein levels produced, correct folding and haem incorporation may have to be met before sufficient levels of active P-450 are generated. The difficulties associated with heterologous expression of a mammalian membrane bound P-450 protein in bacteria may relate to the differences in the structure and compartmentalisation encountered in the procaryotic system whose own endogenous P-450 enzymes are soluble. By employing the yeast *S.cerevisiae* as a host in which to heterologously express mammalian P-450 some of the problems associated with these procaryotic differences may be surmounted.
The aims of expressing Cyp2e1 protein in yeast would be the same as those discussed in connection with procaryotic expression, that is to allow the study of the potential role the CYP2E subfamily plays in the activation and metabolism of a series of common chemicals and carcinogens through the use of mutational assays.

Section 5.7.1: The use of *S. cerevisiae* in mammalian P-450 heterologous expression and mutagenicity testing

S. cerevisiae contain the endogenous endoplasmic attached P-450 molecule $P450_{14DM}$ (CYP51), a lanosterol 14 α-demethylase involved in the synthesis of membrane sterols. This enzyme forms the target for the antifungal properties of ketoconazole and itrakonazole (Yoshida & Aoyama, 1987). *S. cerevisiae* has also been shown to possess a membrane bound NADPH P-450 reductase and cytochrome b_5 protein both of which donate reducing equivalents to P-450 (Hata *et al.*, 1983; Kapelli, 1986). The levels of endogenous P-450 present is dependent on the fermentative state of the yeast; low levels of P-450 are seen when the cells are grown in low concentrations of glucose (below 2.5% w/v) but rise as glucose levels are elevated (Wiseman *et al.*, 1985).
Unlike the procaryotic heterologous expression systems *S. cerevisiae* is capable of recognising and translating mammalian messages usually without the initial need for any modification of a mammalian cDNA sequence. Using this system a variety of mammalian P-450 enzymes have been expressed and these studies have shown that the *S. cerevisiae* recognise the mammalian membrane insertion signal and that the endogenous P-450 support proteins are capable of reducing the heterologous P-450 enzymes (Guengerich *et al.*, 1991).
Mutation assay systems have been established using *S. cerevisiae* as a target for genotoxic damage assessment and a number of chemicals have been studied in this

manner. These systems operate on similar principals to those described in connection with bacterial mutagenicity testing and rely on the chemical damage to produce an assayable mutation within the *S. cerevisiae* and other fungal genomes. An example of a mutation assay system in *S. cerevisiae* is the generation of mutations in the arginine permease gene (*CAN 1*) which leads to resistance to the arginine analogue L-canavanine (Whelan *et al.*, 1979). Mutagenicity assays in *S. cerevisiae* also suffers from the shortfalls associated with the procaryotic mutation systems principal amongst these being that many chemicals tested require extracellular activation and cellular penetration before a mutagenic response is initiated. Extracellular activation systems employing mammalian microsomal fractions have been used in an attempt to overcome this failing. Yeast mutagenicity assay systems were seen to be less sensitive than those employing procaryotes and required much higher minimal concentrations of test chemicals before a mutagenic response was elicited. The reduced sensitivity of yeast to mutagenicity was suggested to relate to the presence of complex endogenous detoxification systems (Cullen & Philpot, 1977; Zimmerman *et al.*, 1984). In a similar manner to that seen using procaryotic systems, many of the nitrosamines tested in *S. cerevisiae* did not generate significant mutagenicity in these systems in spite of their clear carcinogenic actions *in vivo* in mammalian studies (Larimer *et al.*, 1978; Mehta & VanBorstel, 1984).

The intracellular heterologous expression of enzymes which may be responsible for the activation of chemicals in *S. cerevisiae* may circumvent the problems associated with the extracellular generation of the reactive metabolites in a similar manner to that proposed in the procaryotic system(Section 5.5.1). By combining the heterologous expression of rat CYP2B1 and a mutagenicity assay in *S. cerevisiae*, it was shown that the mutagenicity of cyclophosphamide, a chemotherapeutic agent, and sterigmatocystin, a carcinogenic mycotoxin, are elevated in the CYP2B1 expressing cells demonstrating the efficacy of such an approach (Black *et al.*, 1989).

Section 5.7.2: Expression of Cyp2e1 protein in *S. cerevisiae*

The vectors employed to express the Cyp2e1 cDNA in *S. cerevisiae* were episomal and based on the 2μ plasmid, an endogenous plasmid present in most strains at around 100 copies per haploid genome. The 2μ plasmid is modified in the expression vectors by the inclusion of a *S. cerevisiae* gene promoter region, a selectable marker for cells carrying the plasmid and in some cases a terminator region of a yeast gene to allow efficient termination and poly-(A) addition of the heterologous message to occur. The selectable markers employed in the expression vectors often take the form of genes which allow complementation of auxotrophic mutations present in the host cells. Most of the promoters employed in *S. cerevisiae* vectors are derived from highly expressed glycolytic enzymes and can either be constitutively or inducibly transcriptionally activated (Romanos *et al.*, 1992).
Four vectors were used to generate expression of Cyp2e1 in *S. cerevisiae* :

The pMA56 plasmid: This vector contains an alcohol dehydrogenase I (*ADC1* or *ADH1*) promoter which has constitutive transcriptional activity when the host is grown in the presence of glucose. The Cyp2e1 cDNA was cloned into an *Eco*R I restriction endonuclease site downstream of the *ADC1* promoter and the orientation of the insert was established using diagnostic *Bam*H I restriction endonuclease sites. No specific terminator regions were engineered into the vector but transcripts produced from the *ADC1* promoter have been shown to terminate as a result of signals contained within an adjacent interrupted 2μ *FLP* gene which previously encoded a site-specific recombinase. The vector carries the *TRP1* gene and is selected in the absence tryptophan (Ammerer, 1983).

The pVT100U phagemid: This vector contains a slightly smaller fragment of the same alcohol dehydrogenase I gene as used in the pMA56 vector. The Cyp2e1 cDNA was directionally cloned into *Xba* I and *Hind* III restriction endonuclease sites downstream of the promoter. The heterologous transcript is terminated and poly-(A) added as a result of the termination sequence of the *ADH* 1 gene placed downstream from the cloning site. The plasmid carries a *URA* 3 gene and is selected in the absence of uracil (Vernet *et al.*, 1987).

The pYeDP1/10-1 plasmid: This vector contains a phosphoglycerate kinase promoter (*PGK*) which has constitutive transcriptional activity in the presence of glucose. The vector was digested with *Bgl* II restriction endonuclease and the resultant 3' recessed DNA termini were then end-filled to generate flush, "blunt", 3' and 5' termini using

the Klenow fragment. The Cyp2e1 cDNA contains an internal *Bgl*II restriction endonuclease site and also cannot be excised from the pBS SKII(-) plasmid to yield blunt-ends with the restriction sites available. The Cyp2e1 cDNA was released as an *Eco*R I restriction endonuclease fragment which was treated with the Klenow fragment to end-fill the recessed 3' DNA termini generated. The end-filled vector and cDNA were ligated and the orientation of the Cyp2e1 cDNA was determined using a diagnostic *Bam*H I restriction endonuclease site. The transcript is terminated by the inclusion of signals from the downstream region of the *PGK* gene (Cullin & Pompon, 1988). This vector was derived from the pMA91 vector and contains the same promoter and terminator sequences present in the pMA91 plasmid (Mellor *et al.*, 1983; Cullin & Pompon, 1988); the pMA91 vector has been successfully employed to generate the heterologous expression of a variety of mammalian P-450 proteins (Guengerich *et al.*, 1991)

The YepGAL plasmid: This vector contains a hybrid promoter constructed from the *GAL1* and *GAL10* genes which are involved in the metabolism of galactose. This promoter is transcriptionally active only in the presence of galactose; in the absence of galactose the transactivator of the *GAL* genes, GAL4, is bound by the repressor GAL80; in the presence of galactose this interaction is lost and the transactivator binds the promoter and generates transcription. Transcription is initiated from the promoter by growing the host cells in the presence of galactose (Geitz & Sugino, 1988). The Cyp2e1 cDNA was directionally cloned into the *Xba*I and *Hind*III restriction endonuclease sites of the YepGAL vector. The plasmid does not carry a formal transcript terminator region and contains the tryptophan selectable *TRP1* gene. The plasmid was the kind gift of Dr A. Boyd, Department of Biochemistry, University of Edinburgh.

The vectors, relevant restriction endonuclease sites and cloning strategy employed in the generation of these Cyp2e1 constructs is shown in Figure 5.18.

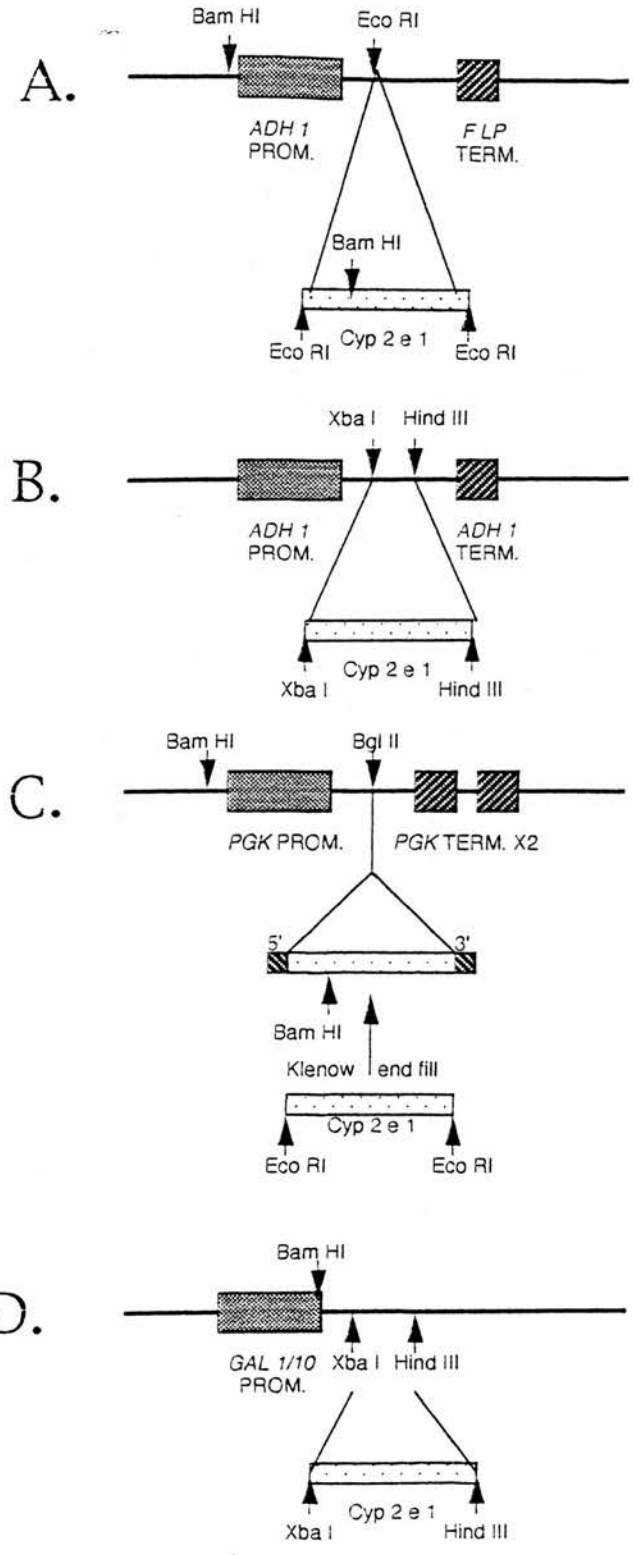


Figure 5.18: The cloning strategies employed to generate the 4 Cyp2e1 constructs for the expression of Cyp2e1 protein in *S. cerevisiae*.. The restriction endonuclease sites in the vector and inserts used for cloning are illustrated as are the *Bam* H I sites which allowed the orientation of the insert within the vector. **PROM:** Promoter **TERM:** Terminator. The diagram is not to scale and the vectors are circular.

A: pMA56. **B:** pVT100U. **C:** pYeDP1/10. **D:** YepGal.

The constructs were used to transform *S. cerevisiae* strains and the relevant auxotrophic selection applied. Cells were also transformed with the parental vector alone and if available the construct containing the Cyp2e1 cDNA in the reverse orientation. The pMA56, pVT100U, and YepGAL constructs were used to transform the *S. cerevisiae* KY118 strain which has been employed previously in mutagenicity assays. This strain has been shown to possess very low levels of endogenous P-450 which may otherwise potentially interfere with the activation of mutagenic chemicals (Black *et al.*, 1989). The pYeDP1/10-1 construct was used to transform the *S. cerevisiae* W3031B strain which has previously been seen to generate P-450 protein expression in combination with this vector (Cullin & Pompon, 1988). As all the constructs were selectable either in the absence of tryptophan or uracil the growth medium could be supplemented by the addition of the acid protein hydrolysates casamino acids (CAA); CAA lack these nutrients and their inclusion in the culture medium increases the growth rate and protein synthesis of *S. cerevisiae*. Cells were harvested from these cultures when they obtained an optical density at 600 nm (OD_{600}) of 0.4, 0.6, 0.8 and 1.0 which were taken to represent early, mid and late logarithmic, and stationary growth phases respectively. Total cellular protein was prepared after the cells were disrupted using glass-beads and samples containing equal protein concentrations were subjected to Western blotting and the potential heterologous expression of Cyp2e1 protein assessed using the polyclonal antibody raised against purified rat CYP2E1 protein. Cyp2e1 protein could only be detected in the cells transformed with the pMA56 construct and the highest concentration was observed at an OD_{600} of 0.8 (Figure 5.19).

These results emphasise the stochastic nature associated with the expression of a novel cDNA under the control of various promoter constructs. In each instance the information contained within the heterologous transcript encoding the Cyp2e1 protein itself is virtually identical. However, differences in the nature of the recombinant insert arise however outside the coding region as a result of the cloning strategy employed and these changes generate messages with different leader sequences which have been suggested to be important determinants in the efficiency of translation (Cullin & Pompon, 1988). The observed differences in Cyp2e1 protein levels also could relate to the differences in the transcription levels produced by the various promoters and the nature of the terminator of the heterologous transcript and resultant message stability. The observation that the pMA56 *ADC1* promoter (Ammerer, 1983), which produced detectable levels of Cyp2e1 protein, was just a slightly larger version of the promoter present on the pVT100U (Vernet *et al.*, 1987), which did not produce detectable Cyp2e1 protein levels, underlines not only how great a role these variables must play in the generation of successful heterologous expression but also how the expression of a

novel cDNA to a large extent must still be viewed as an empirical exercise. Subsequent analysis was performed only on the pMA56/Cyp2e1 construct.

Section 5.7.3: Subcellular localisation, spectral and activity analysis of the pMA56/Cyp2e1 generated Cyp2e1 protein in *S. cerevisiae*

Cellular fractions of the *S. cerevisiae* KY118 strain transformed with pMA56/Cyp2e1 and expressing Cyp2e1 protein were prepared to establish the subcellular localisation of the Cyp2e1 protein. Cells were harvested at an OD_{600} of 0.8, disrupted by passage through a French press, and then separated into crude fractions by density centrifugation according to a modification of the method of Yoshida and Aoyama (1984). An initial centrifugation step at 11,000g removed the cellular debris, unbroken cells, cell walls and larger organelles such as mitochondria. The supernatant was then centrifuged at 100,000g to pellet a membrane enriched fraction, the resultant supernatant was taken as a cytosolic enriched fraction. The distribution of the Cyp2e1 protein in these fractions was assayed by Western blotting using the polyclonal antibody raised to purified rat CYP2E1 protein. The Cyp2e1 protein was localised in the membrane enriched and debris fraction with no detectable protein present in the cytosolic fraction (Figure 5.20).

Reduced carbon monoxide difference spectra were performed on the membrane enriched fraction from the *S. cerevisiae* KY118 strain transformed with pMA56/Cyp2e1 in both the correct and reverse orientation. A small amount of haem incorporation was detected producing an absorbence peak at 450 nm present in cells expressing Cyp2e1 protein which was absent in the membrane fractions of the cells which did not (Figure 5.21). The KY118 *S. cerevisiae* , a strain which has a virtually undetectable endogenous P-450 population, would not be expected to contain high levels of endogenous P-450 at the glucose concentrations in which the cells were grown and most other endogenous cytochrome components would be expected to be localised in the mitochondrial containing fraction (Black *et al.*, 1989). A peak at 420 nm was also produced in the membrane fraction expressing Cyp2e1; in general, in P-450 studies, it is not clear what the peak at 420 nm represents but it is thought to indicate the disturbance of the haem:apoprotein interaction as the P-450 begins to lose the haem moiety (Eliasson *et al.*, 1990), suggesting that problems with haem insertion or maintenance may still be present. A low level of haem incorporation compared to the level of apoprotein generated is a common problem in the heterologous expression of P-450 in *S. cerevisiae* (Oeda *et al.*, 1985; Murakami*et al.*, 1986; Murakami*et al.*, 1987; Fujita *et al.*, 1990).

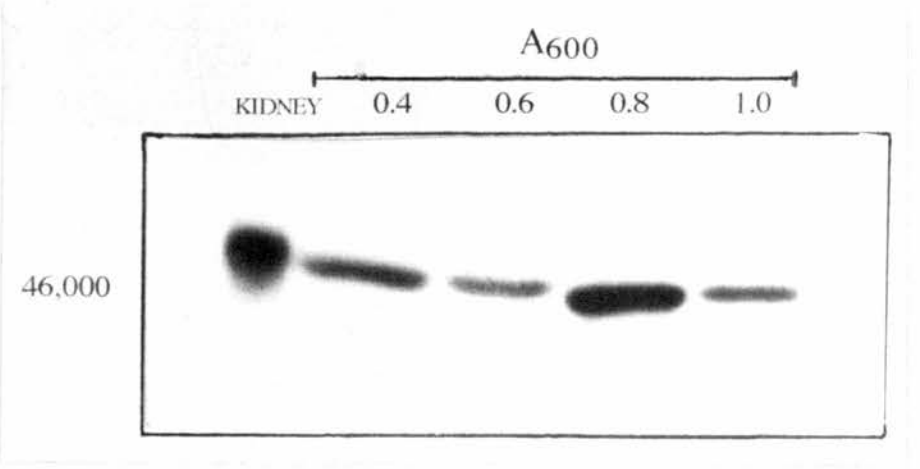


Figure 5.19: The expression of Cyp2e1 protein in *S. cerevisiae* KY118 from the pMA56 vector detected using polyclonal antisera raised against purified rat CYP2E1. Cells were harvested on obtaining an OD_{600} (**A_{600}**) as indicated, and disrupted using glass beads. 50μg of total cellular protein was loaded per track as well as 10μg of male mouse kidney microsomal fraction.

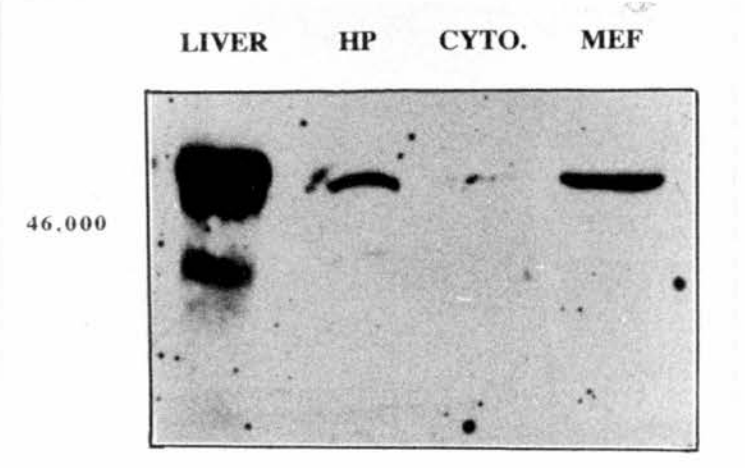


Figure 5.20: The subcellular localisation of Cyp2e1 protein in *S. cerevisiae* KY118 produced from the pMA56 vector detected using polyclonal antisera raised against purified rat CYP2E1. The cells were harvested at an OD_{600} of 0.8, disrupted by French pressing, and separated by centifugation into fractions enriched in unlysed cells, large organelles, etc. (**HP**), membrane enriched (**MEF**), and cytosolic protein (**CYTO.**); 50μg of each fraction were loaded per track as well as 5μg of male mouse liver microsomal fraction.

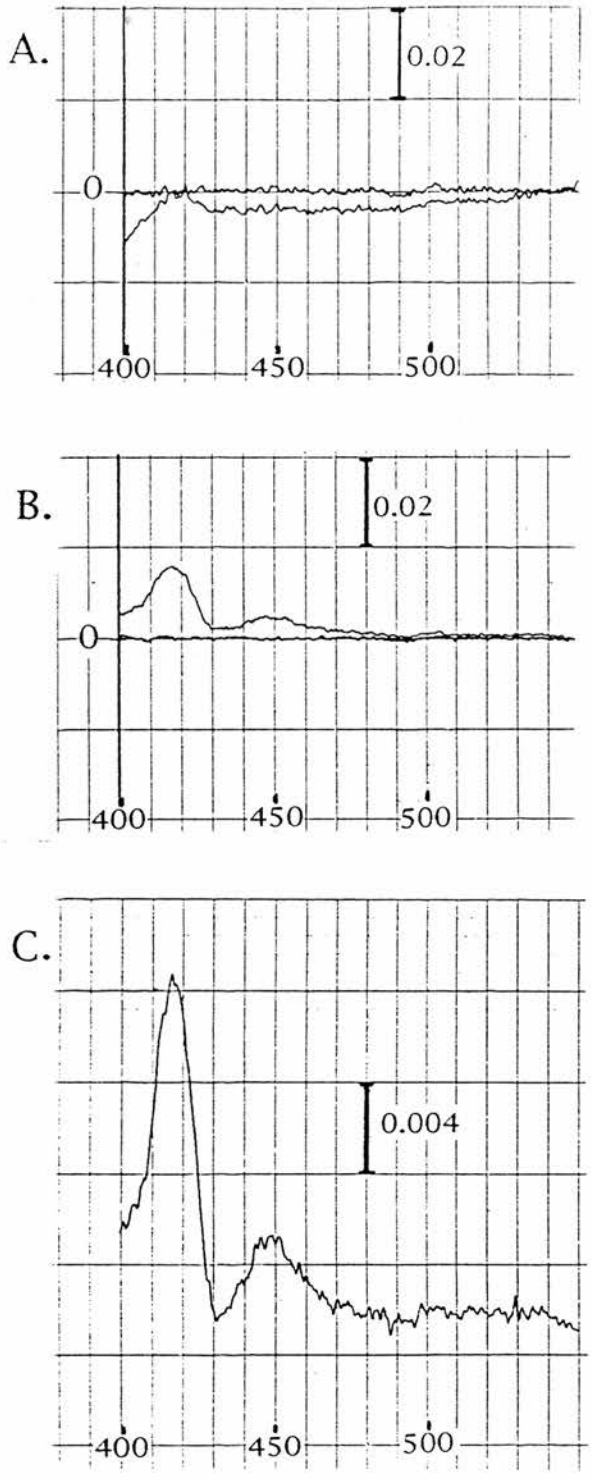


Figure 5.21: Analysis of the Cyp2e1 protein heterologously expressed in *S. cerevisiae* KY118 for haem content by reduced difference spectroscopy. **A:** The reduced difference spectra produced in the membrane enriched fractions (2mg/ml) of *S. cerevisiae* KY118 transformed with the pMA56 plasmid alone. **B:** The reduced difference spectra produced in the membrane enriched fractions (2mg/ml) of *S. cerevisiae* KY118 transformed with the pMA56/Cyp2e1 construct. **C:** A stored trace of **B.** with a reduced scale; in both **A.** and **B.** peaks at 450nm and 420nm are produced indicative of haem content within the Cyp2e1 protein.

The levels of Cyp2e1 protein present in the membrane enriched fraction by *S. cerevisiae* were low compared to those present in a much smaller total protein amount from male mouse kidney. This was illustrated by the comparison of the level of rat CYP2B1 protein generated using the pMA56 vector and which was shown to produce detectable enzymatic activity. The pMA56/rat CYP2B1 construct (kind gift, Dr S. M. Black, Department of Paediatric Medicine, UCSF, CA.) was used to transform the *S. cerevisiae* KY118 strain and total cellular protein prepared; by loading equal amounts of protein from *S. cerevisiae* expressing CYP2B1 and Cyp2e1 and equal amounts of rat and mouse total microsomal liver fractions the levels of P-450 protein produced relative to the levels present in the liver were analysed (Figure 5.22). It can be seen that, given the limitations of the comparison, proportionately a much higher level of CYP2B1 protein was produced.

The catalytic activity of the membrane enriched fraction was assayed using *p*-nitrophenol as a substrate (Reinke & Moyer, 1985; Koop *et al.*, 1989); the membrane enriched fraction containing Cyp2e1 did not generate a significant increase in *p*-nitrophenol hydroxylation above the control membrane enriched fraction or the background activity rate, both of which were around 100 fold less than that produced by an equal amount of mouse liver microsomal material.

Section 5.7.4: Possible methods of increasing the expression levels of Cyp2e1 protein in *S. cerevisiae*

Cyp2e1 protein is expressed in *S. cerevisiae* using this system and detectable levels of haem incorporation occur; the lack of detectable Cyp2e1 activity in the membrane enriched fraction containing Cyp2e1 probably relates to the low absolute levels of active protein present compared to the sensitivity of the assay. It is likely therefore that if the absolute levels of Cyp2e1 protein could be elevated, and assuming that a concomitant increase in the proportion of Cyp2e1 enzyme with correctly incorporated haem resulted, activity could be detected and the system could be employed in mutagenicity testing.

Several approaches may enable an elevation in the level of Cyp2e1 produced to be achieved. As was illustrated by the initial empirical approach using four vectors to establish detectable levels of Cyp2e1 the generation of increased Cyp2e1 protein levels is arguably first to get a system that operates and then to modify that system until higher levels of expression are achieved.

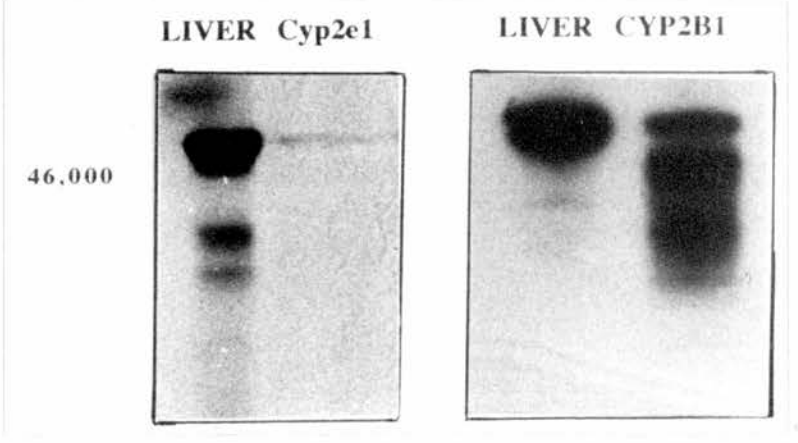


Figure 5.22: A comparison of the levels of expression of mouse Cyp2e1 and rat CYP2B1 protein in *S. cerevisiae* KY118 from the pMA56 vector. In the left-hand box the level of Cyp2e1 present in 5μg of male mouse liver microsomal fraction (**LIVER**) is compared to 5μg of *S. cerevisiae* KY118 total cellular protein (**Cyp2e1**) using the polyclonal antibody raised against purified rat CYP2E1. In the right-hand box the level of CYP2B1 present in 5μg of rat liver microsomal fraction (**LIVER**) is compared to 5μg of *S. cerevisiae* KY118 total cellular protein (**CYP2B1**) using the polyclonal antibody raised against purified rat CYP2B1. In this sample, as discussed in Black, *et al.* , (1989), it is seen that degradation of the CYP2B1 protein in *S. cerevisiae* is occurring.

S. cerevisiae possess a codon bias in its transcripts which has been shown to lead to not only low levels of heterologous protein translation, but also low levels of heterologous transcript production. The level of translation of a protein directly relates to the level of codon bias present in its transcript, thus proteins present in the cell at a high concentration such as alcohol dehydrogenase I has a message which uses 90% preferential codons, whereas a low concentration protein such as iso-2-cytochrome c possesses a preferential codon usage of 20% (Bennetzen & Hall, 1981). This control mechanism has been demonstrated using mutations of the phosphoglycerate kinase (PGK) protein; normally PGK is present at between 20% and 30% of the total cellular protein but this level was reduced to just detectable levels as modifications in the codon bias present in the message were introduced. These mutations were seen to be particularly effective in the portion of the transcript encoding the N-terminal region of the protein (Hoekema *et al.*, 1987). The codon usage in the coding regions of a message also affects the transcriptional activity of the promoter, and downstream activating sequences (DAS) have been discovered in a variety of transcripts including those of *PGK* (Mellor *et al.*, 1987), lipoamide dehydrogenase and pyruvate kinase (Zaman *et al.*, 1992).

Although ostensively the *S. cerevisiae* translational machinery can recognise the translational initiation sequences of mammalian transcripts, the consensus surrounding the initiation codon differs which affects translational efficiency and leads to a reduction in the amount of heterologous protein produced (Hamilton *et al.*, 1987. Table 5.5).

Table 5.5: The consensus sequences surrounding the initiation codon in yeast and higher eucaryotes where 5 indicates the initiation codon.

	1	2	3	4	5	6
YEAST	$^{A}/_{C/U}$	A	$^{A}/_{U}$	A	**AUG**	UCU
HIGHER EUCARYOTES	C	A	C	C	**AUG**	G

The sequence surrounding the initiation codon in *S. cerevisiae* was seen to be high in adenosine and uracil residues and is not predicted to be capable of generating any strong secondary structures. The introduction of such structural elements, as present in many mammalian transcripts and potentially the CYP2E subfamily (Figure 5.7a), into the *PGK* transcript dramatically reduced the translational level and this factor may lead to low levels of translation of mammalian transcripts in *S. cerevisiae* (van den Heuvel *et al.*, 1990).

Modifying the codon bias of the Cyp2e1 cDNA and tailoring the context of the initiation codon to be more easily translated by the *S. cerevisiae* in order to generate higher levels of translation, and introducing other changes to the cDNA such as DAS regions to increase the levels of transcripts produced, would require either a significant amount of site directed mutagenesis or the generation and ligation of several mutated Cyp2e1 cDNA fragments. One approach which may however allow the exploitation of the potential benefits of these modifications in transcriptional and translational terms without the need for such a considerable investment of time, would be to fuse the N-terminal transmembrane spanning region of the *S. cerevisiae* endogenous P-450_{14DM} (CYP51) to the Cyp2e1 cDNA. This would change both the initiation codon context and potentially generate a more favourable codon bias in the N-terminal region of the protein. The N-terminal membrane spanning region has previously been shown not to participate in the generation of the specific metabolic activity of the particular isoform (Cullin & Pompon, 1988; Larson *et al.*, 1991) and so would not be expected to lead to changes in either the nature or metabolic parameters of the reactions catalysed by Cyp2e1 protein. This approach leading to the generation of a fusion between a mammalian protein and its yeast counterpart was undertaken in the heterologous expression of the mammalian P-450 reductase in *S. cerevisiae* ; the replacement of the mammalian membrane spanning region with the *S. cerevisiae* endogenous P-450 reductase N-terminal region led to an increase in expression and stability of the heterologously produced protein (Murakami *et al.*, 1986; Murakhami *et al.*, 1987; Bligh *et al.*, 1991). By engineering a fusion between the CYP51 and Cyp2e1 cDNA sequences in the pMA56 expression system developed it is possible that high enough levels of active Cyp2e1 protein could be produced to allow the testing of CYP2E subfamily substrates in a *S. cerevisiae* mutagenicity assay system.

Section 5.8: The Cyp2e1 gene

Cloning the Cyp2e1 gene would allow analysis of the potential control regions both within the regions 5' of the transcriptional start site as well as further analysis of the nature of the β2 SINE insertion within the 3' UTR. It would allow a comparison of the gene structures of the Cyp2e1 gene and other characterised CYP2E subfamily genes and establish the levels of intervening sequence homology; a knowledge of the Cyp2e1 gene may also allow the potential manipulation of the mouse genome through the use of gene targeting strategies. It was decided that the Cyp2e1 gene would be characterised pragmatically and that only those portions required to enable further studies to be carried out would be completely analysed; no attempt was made to completely sequence all of the intervening sequences of the Cyp2e1 gene.

Section 5.8.1: Cyp2e1 gene copy number

The CYP2E1 gene was seen to be single copy in the rat and human genomes (Song et al., 1986); in the rabbit however there were two genes, CYP2E1 and CYP2E2 (Khani *et al.*, 1987a; Khani *et al.*, 1987b). The two rabbit genes were 97% identical over their coding regions suggesting that, on the basis that a 1% amino-acid sequence divergence occurs every 3.4 to 3.8 million years, that a gene duplication event in the rabbit occurred around ten million years ago. The speciation of the rabbit was thought to have occurred around 60 million years ago (Fitch & Langley, 1976) suggesting that the rabbit CYP2E1 gene probably duplicated long after the speciation of the rabbit and so may not be expected to be seen in other species (Khani *et al.*, 1987a).

In order to evaluate the gene copy number of the Cyp2e1 gene in the mouse, genomic DNA was isolated from DBA/2N and C57BL/6 livers, subjected to restriction endonuclease digestion, electrophoresed on an horizontal agarose gel and analysed by Southern blotting. The resultant filter was hybridised with a radiolabelled probe constructed from the 800 base-pair partial Cyp2e1 cDNA cloned from the λgt11 library (Section 5.2). A simple banding pattern was observed suggesting the presence of only one Cyp2e1 gene in the mouse (Figure 5.23).

The Cyp2e1 gene had been chromosomally localised to chromosome 7 using the a rat CYP2E1 cDNA as a probe in mouse-hamster somatic cell hybrids (Umeno *et al.*, 1987b). The mouse Cyp2a (Kimura *et al.*, 1989) and Cyp2b subfamily (Simmons *et al.*, 1985) clusters have previously been located in this manner to chromosome 7. Not all the mouse family 2 members are located on chromosome 7 however, for example the Cyp2c (Meehan *et al.*, 1988) and Cyp2d (Gonzalez *et al.*, 1987) subfamies have been shown to be present on chromosomes 19 and 15 respectively suggesting that evolution may have spread the subfamily 2 genes throughout the genome.

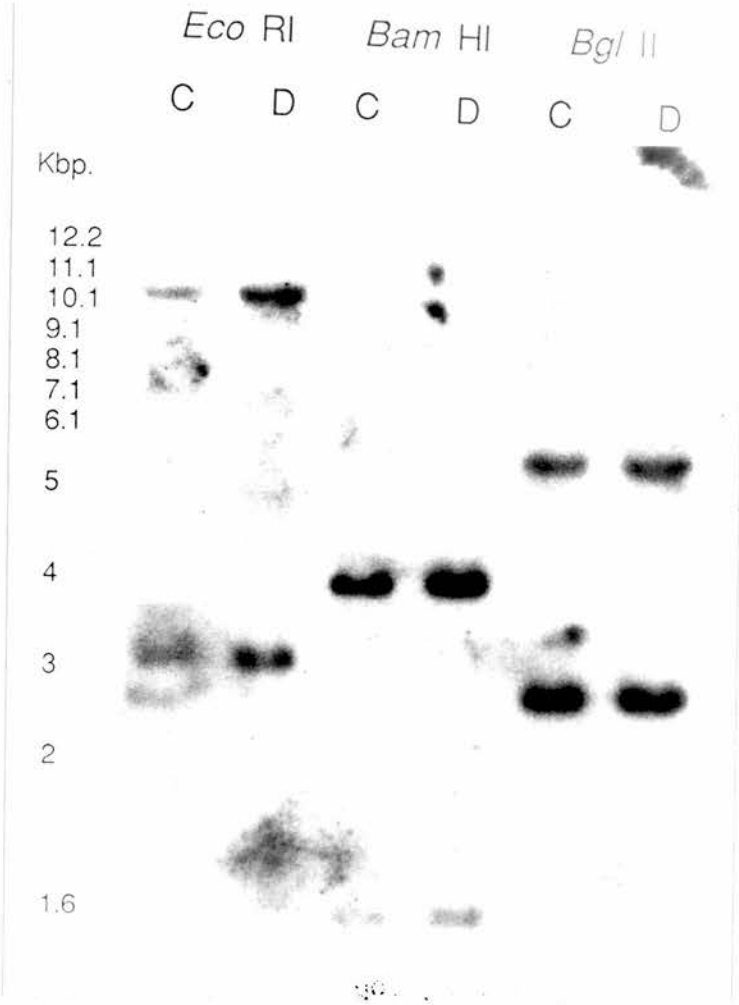


Figure 5.23: Southern analysis of the Cyp2e1 gene. Genomic DNA isolated from C57BL/6 (**C**) and DBA2/N (**D**) mice was subjected to restriction endonuclease digestion using the enzymes indicated, separated on an agarose gel, and transferred to a nylon membrane. The membrane was then hybridised with the 800bp partial Cyp2e1 cDNA probe; DNA marker sizes are indicated.

Section 5.8.2: Cloning and characterising the mouse Cyp2e1 gene

Using the 800 base-pair Cyp2e1 partial cDNA fragment (Section 5.2) as a probe several clones were isolated from a BALB/c mouse liver genomic library constructed in the bacteriophage lamda vector λEMBL3, a replacement vector capable of containing a recombinant fragment of up to twenty kilobases of genomic DNA (Frischauf *et al.*, 1983). Three clones were subjected to further rounds of screening until plaque pure genomic phage clones hybridising to the Cyp2e1 cDNA were obtained. Phage DNA from one of the recombinant mouse genomic clones was prepared and subjected to restriction endonuclease digestion with *Sal* I, *Bam*H I, *Xba*I and *Hpa*I and analysed by Southern blotting. The filter was hybridised with the 800 base-pair Cyp2e1 partial cDNA clone used initially to clone the λEMBL3 recombinant phage. Four reactions were set up with increasing amounts of restriction endonuclease for each digestion to ensure that a complete digestion pattern was obtained. The results from this analysis are shown in Figure 5.24. The recombinant phage clone was seen to contain an approximately five kilobase-pair *Sal* I endonuclease fragment hybridising to the 800 base-pair fragment probe which was isolated from an agarose gel and subcloned into the plasmid vector pUC19. The *Sal* I fragment was selected for subcloning as the restriction endonuclease is resistant to the high salt concentrations present in the phage DNA preparations making it unlikely that the *SalI*I fragment is the result of a partial digestion of the phage DNA which clearly is a problem with some of the restriction endonucleases used in the analysis of the phage DNA (Figure 5.24).

Section 5.8.3: Restriction analysis of the five kilobase-pair recombinant *Sal*I fragment hybridising to the 800 base-pair Cyp2e1 probe

Restriction endonuclease digestion analysis of the 5 kilobase-pair *Sal*I fragment was undertaken in order to generate smaller portions of the whole fragment for sequencing. By using restriction endonucleases with a known cutting frequency within the plasmid vector pUC 19 the cutting frequencies within the recombinant fragment could be assessed (Figure 5.25). It was seen that the fragment contained two of the *Hpa*I sites (a restriction endonuclease generating a blunt ended DNA product) suggested from the initial Southern analysis of the clone. pUC 19 has no *Hpa*I sites, and so the whole fragment could be divided into three smaller pieces (approximately 2.8, 1.8 and 0.7 kilobase-pair fragments) by using both *Hpa*I and *Sal*I (Figure 5.25).

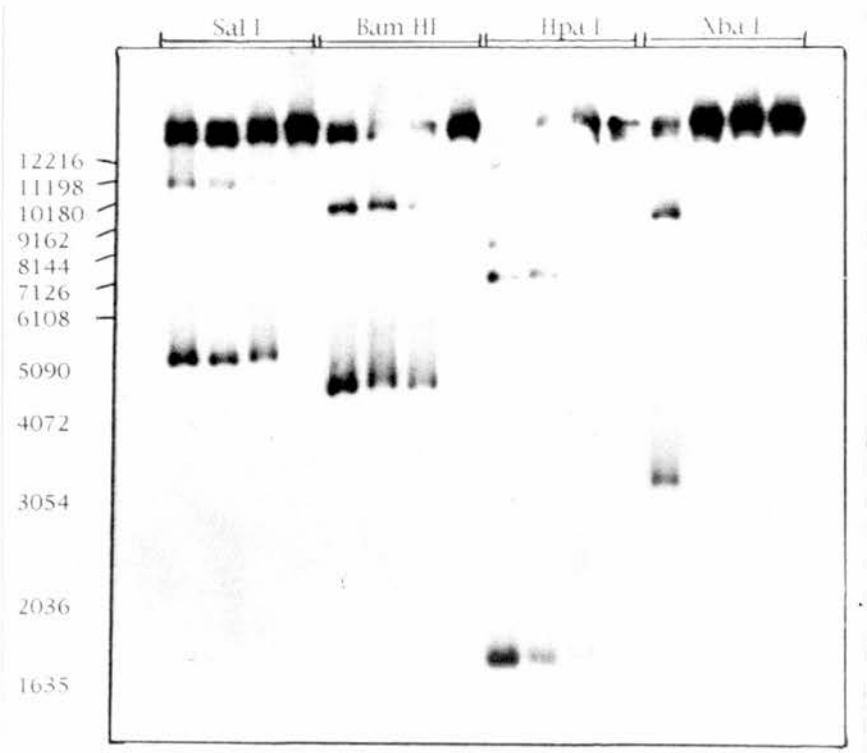


Figure 5.24: Southern analysis a λEMBL3 recombinant clone containing an insert hybridising to the 800bp partial Cyp2e1 cDNA probe. The λEMBL3 DNA was subjected to restriction endonuclease digestion as indicated with (from right to left) 1, 2, 5, and 10 units of the respective restriction endonuclease to ensure that complete digestion was obtained. The DNA was separated on an agarose gel, transferred to a nylon membrane and hybridised with the 800bp partial Cyp2e1 cDNA probe. DNA marker sizes are indicated.

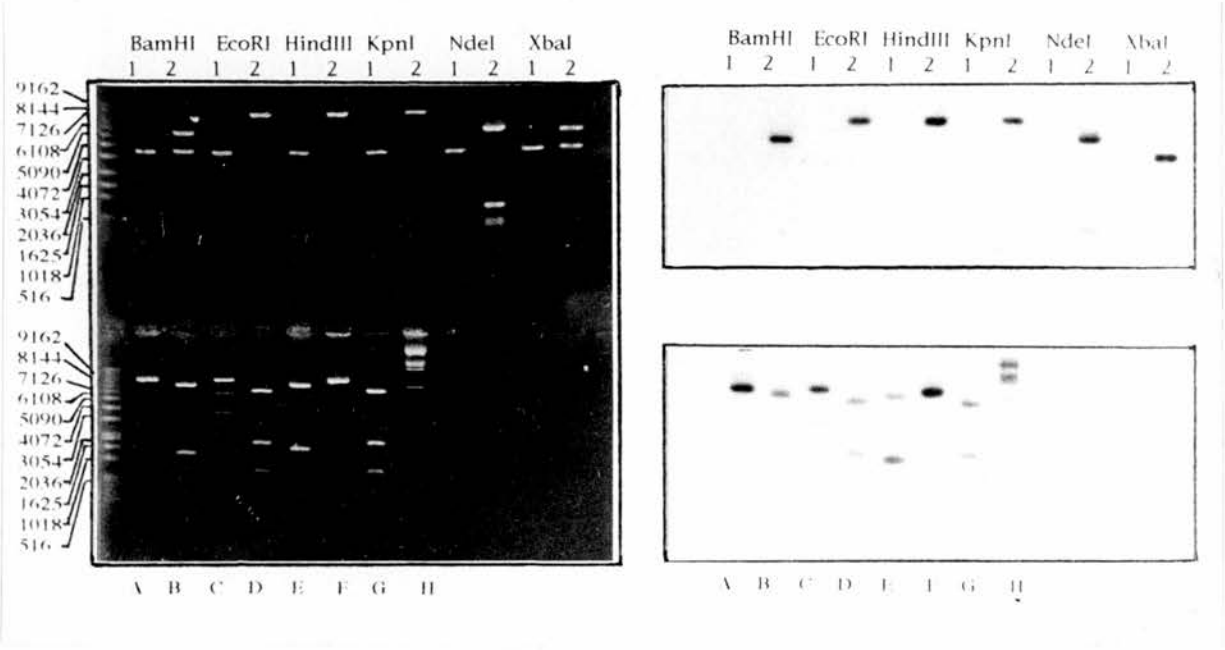


Figure 5.25: Restriction endonuclease analysis of the 5 Kbp *Sal* I Cyp2e1 fragment subcloned into pUC 19. In the upper half of the Figure restriction endonucleases, as indicated, with a single site in pUC 19 were used; in the lower half of the figure restriction endonucleases with no sites in pUC 19 were used (**A:** *Eco* RV **B:** *Apa* I **C:** *Stu* I **D:** *Nco* I **E:** *Hpa* I **F:** *Cla* I **G:** *Nco* I **H:** *Xho* I). The DNA was separated on an agarose gel, transferred to a nylon membrane and hybridised with the 800bp partial Cyp2e1 cDNA probe; DNA marker sizes are indicated. From this analysis it was seen that the 5Kbp fragment could be restricted to produce 3 smaller fragments following digestion with *Sal* I (the original cloning restriction endonuclease) and *Hpa* I.

The two smaller *Hpa*I and *Sal*I 0.7 and *Hpa*I/*Hpa*I 1.8 kilobase-pair fragments were prepared and subcloned into *Sma*I (another restriction endonuclease generating a blunt ended DNA product so complementary to the *Hpa*I generated DNA ends) and *Sal*I respectively digested M13mp18 and the complementary M13mp19 replicative form DNA. The filamentous bacteriophage M13 vectors operate most efficiently with insert sizes of a maximum of around two kilobase-pairs; DNA fragments larger than this are both difficult to subclone, affect the bacteriophage replication, and can be subject to recombinational deletion (Messing *et al.*, 1983). The larger *Hpa*I and *Sal*I 2.8 kilobase-pair fragment, too big to be successfully analysed in M13,was subcloned into the phagemid vector pTZ18 R and pTZ19 R (Pharmacia) which have inverted polycloning sites and allows single-stranded DNA to be produced as described in the rescue of single-stranded DNA from the pBS II (–) phagemid (Section 5.2.1). Single -stranded DNA was prepared from all of the subcloned fragments and partially sequenced to allow the assessment of the position of the five kilobase-pair fragment as a whole as well as the relative positioning of the three smaller sub-fragments in the Cyp2e1 gene by comparison to the known human and rat CYP2E1 gene sequences. The five kilobase-pair fragment was seen to encode the three prime portion of the mouse Cyp2e1 gene with the smaller *Hpa* I and *Sal* I 0.7 and *Hpa* I/*Hpa* I 1.8 kilobase-pair fragments containing intron six and intron six/exon seven/intron seven respectively and the larger *Hpa* I and *Sal* I 2.8 kilobase-pair fragment intron seven, exons eight and nine and downstream beyond the insertion site of the β2 repetitive element. Using this information, exon primers relevant to the gene area were used to enable double-stranded sequencing. The results from these studies, the sequences and the sequencing strategies used are shown in Figures 5.26, 5.27a and 5.27b. The area of the gene surrounding the β2 repeat insertion site is discussed in Section 5.4.

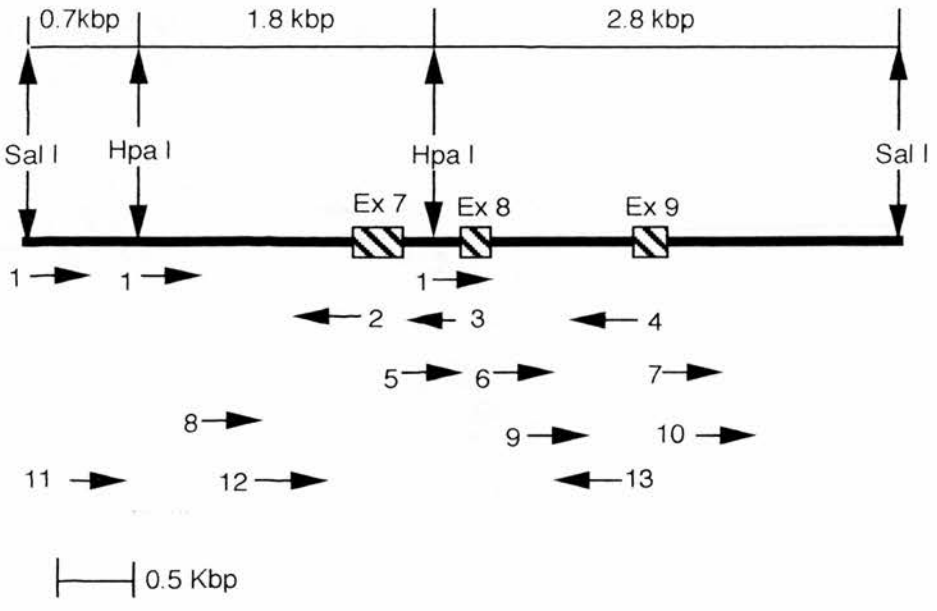


Figure 5.26: The sequencing strategy and oligonucleotides employed to characterise the 5 Kbp *Sal* I fragment containing the 3 ' portion of the Cyp2e1 gene. The *Hpa* I / *Hpa* I and *Hpa* I / *Sal* I portions of the whole 5 Kbp fragment are shown as are the positions and numbers of the exons (**Ex**).

Oligonucleotide sequences

1: Reverse sequencing primer
2: Exon 7, 5 ' (PCR gene structure)
3: Exon 8, 5 ' " "
4: Exon 9, 5 ' " "
5: Exon 7, 3 ' " "
6: Exon 8, 3 ' " "
7: Exon 9, 3 ' " "
8: GAAGATTTCCAAGACCC
9: AGAGTTCTGGGTGGATT
10: Oligo. 10 (cDNA sequencing)
11: AGCTACTGCATACAAAG
12: AGATATGTAGGTGAATG
13: CTCGAGGTGACGTATTT

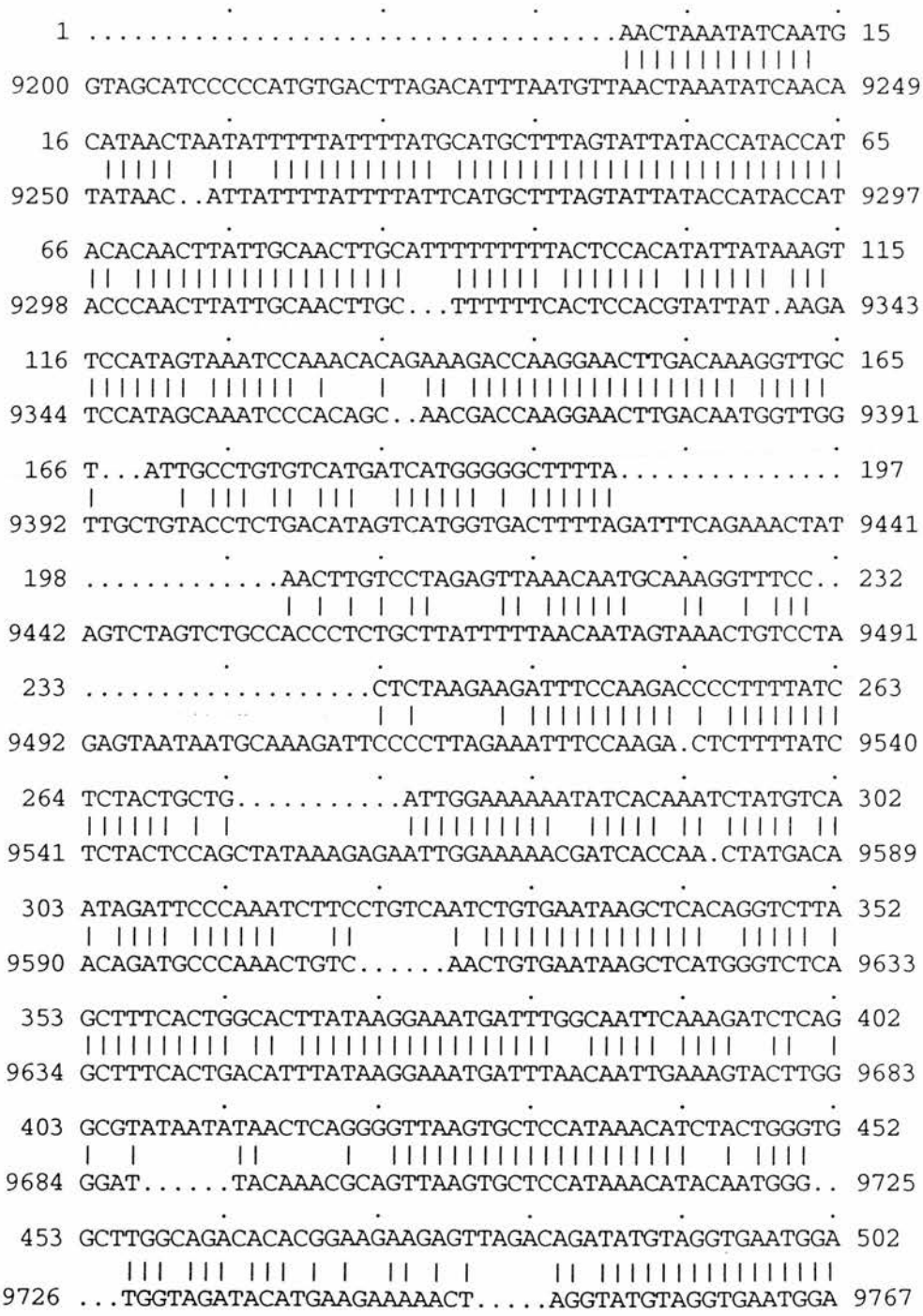


Figure 5.27a: The DNA sequence for the 3 ' portion of the Cyp2e1 gene present in the 1.8 Kbp *Hpa* I / *Hpa* I and part of the 2.8 Kbp *Hpa* I / *Sal* I pieces of the 5 Kbp *Sal* I fragment. The mouse sequence (upper) was compared to the rat CYP2E1 gene sequence (lower) using the GCG Gap alignment program and the gapped similarity between the two sequences is 81.53%. The exons are boxed and the β2 element in the Cyp2e1 3 ' UTR is underlined; also boxed is the poly-A addition site of the rat CYP2E1 mRNA.

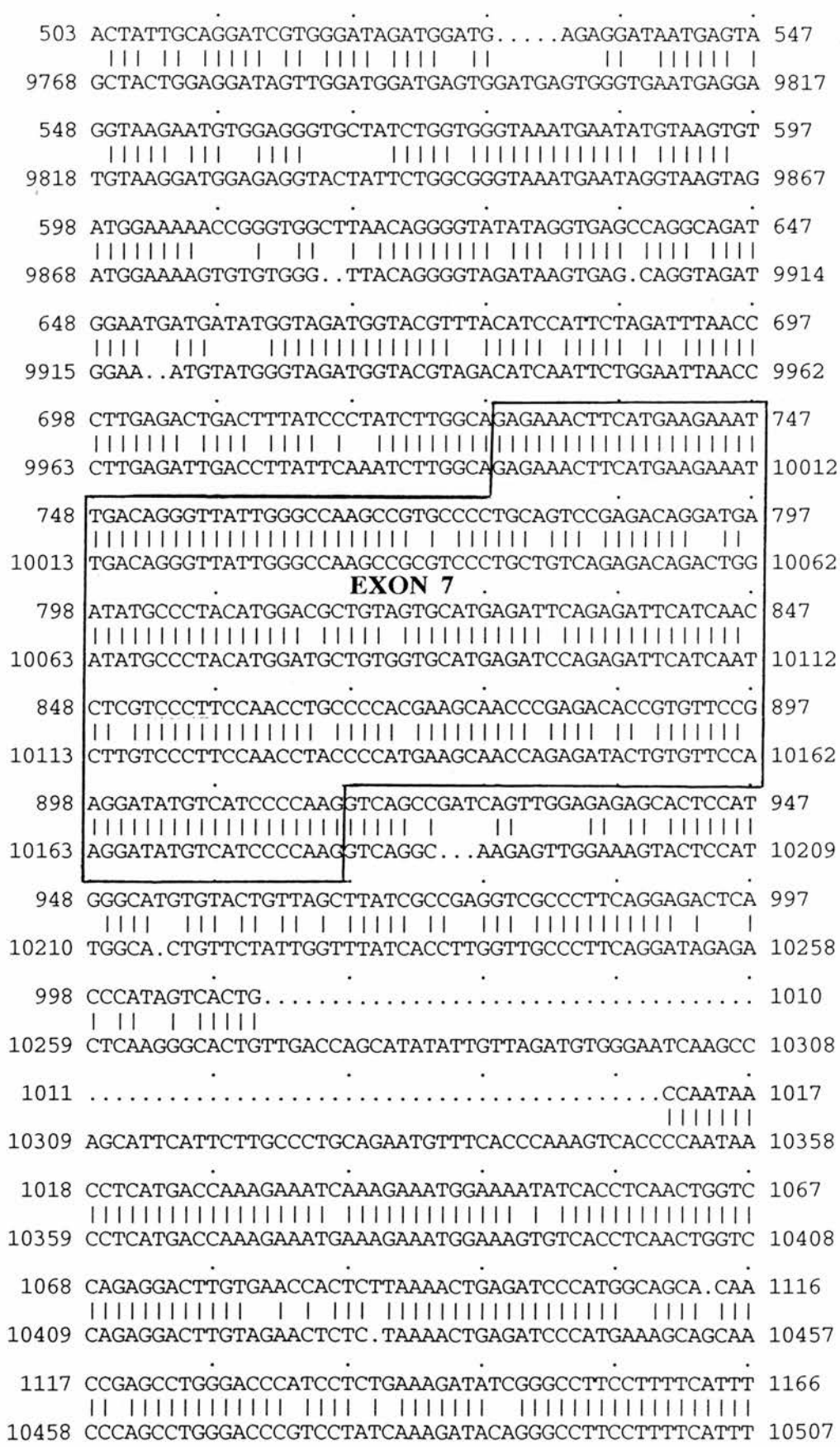


Figure 5.27a (continued)

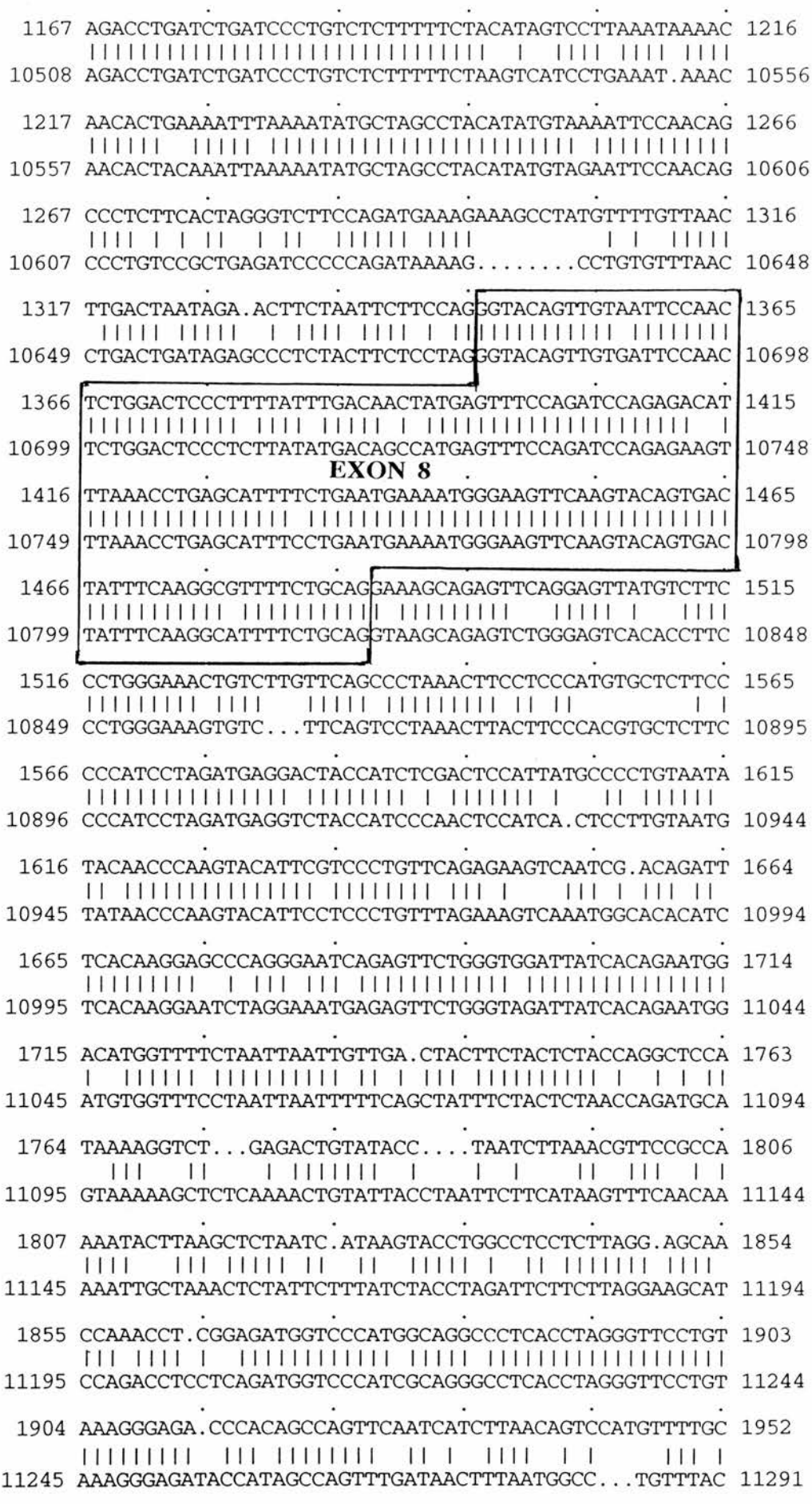


Figure 5.27a (continued)

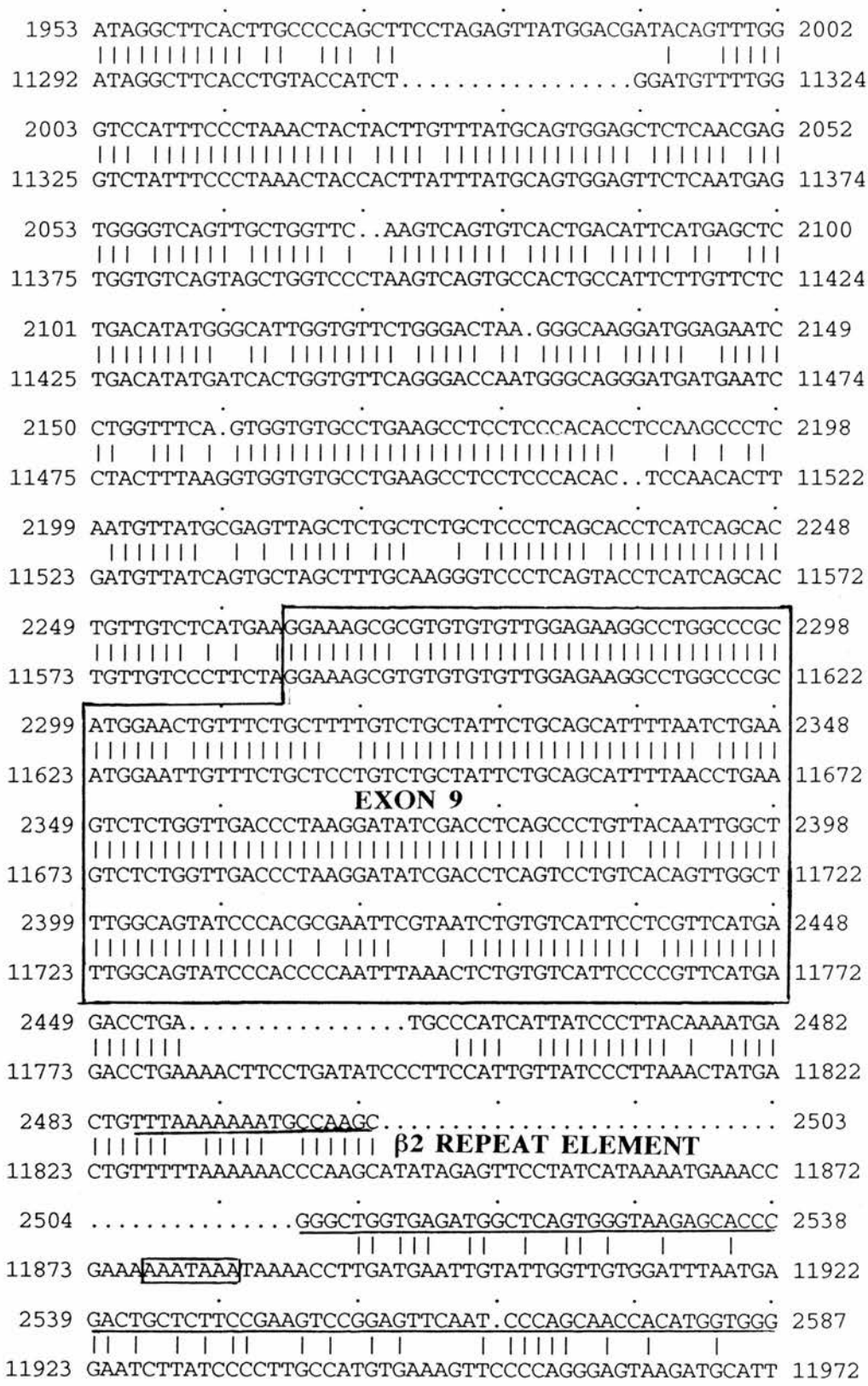


Figure 5.27a (continued)

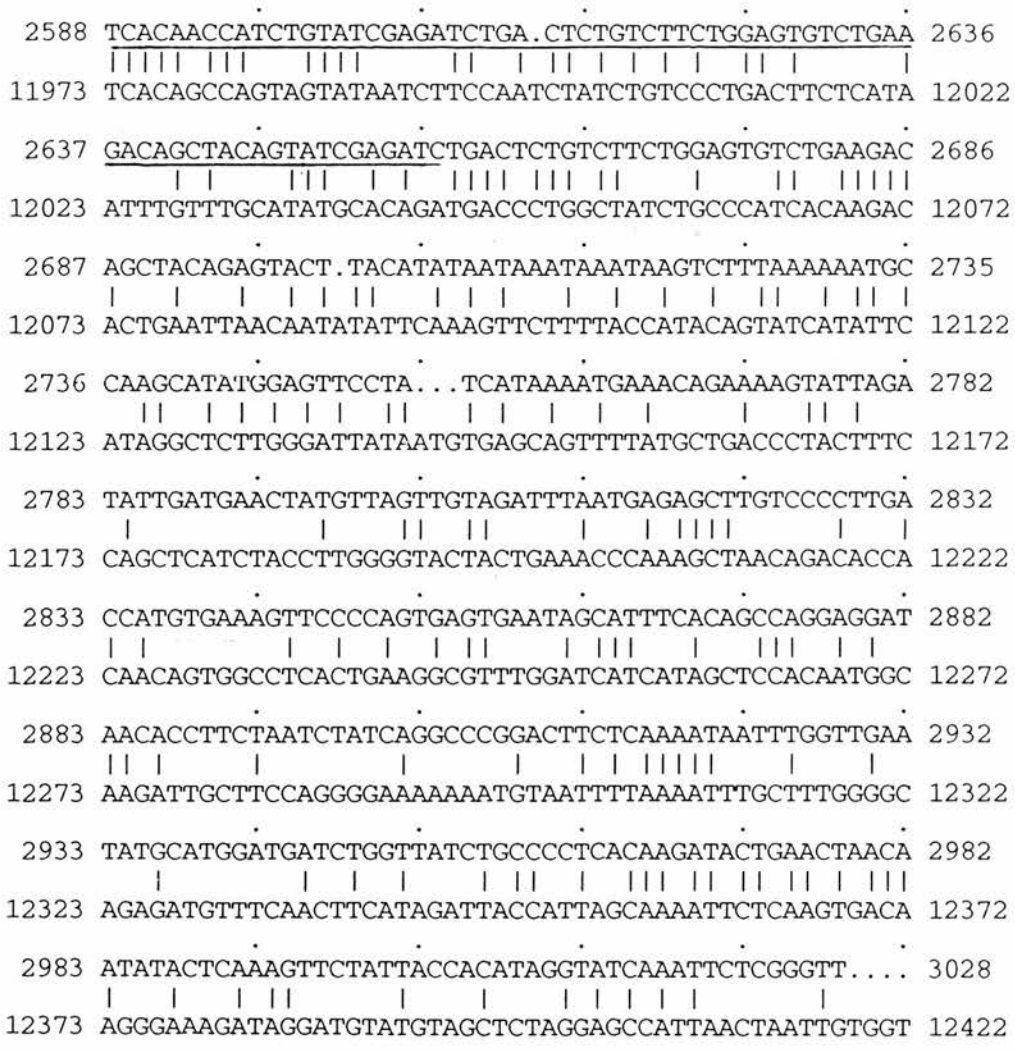


Figure 27a (continued)

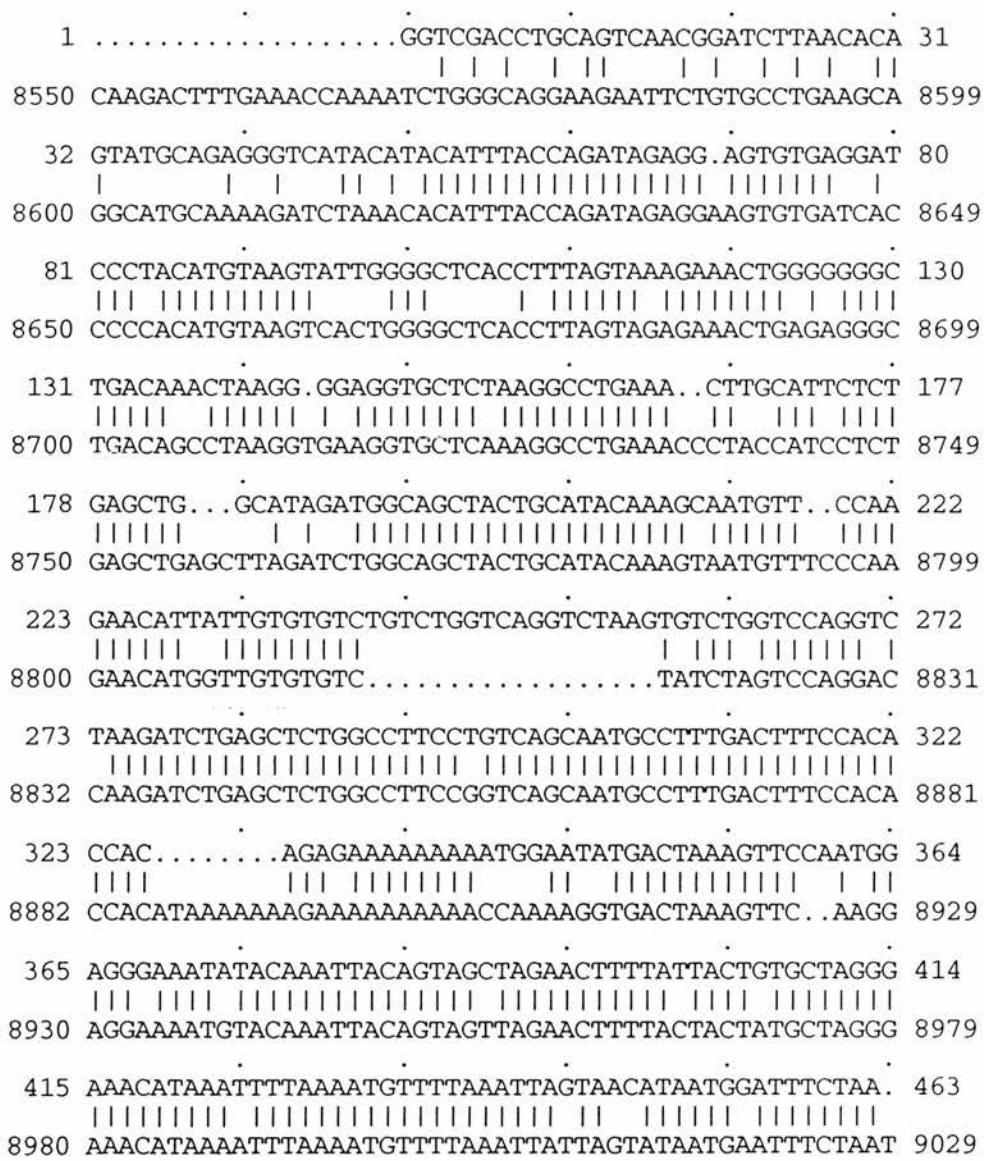


Figure 5.27b: The DNA sequence for the 3 ' portion of the Cyp2e1 gene present in the 1.8 Kbp *Sal* I / *Hpa* I 0.7 Kbp fragment of the 5 Kbp*Sal* I fragment. The mouse sequence (upper) sequence was compared to the rat CYP2E1 gene sequence (lower) using the GCG Gap alignment program and the gapped similarity between the two sequences is 80.36%. The sequence encodes an intronic portion of the upstream from exon 7.

5.8.4: Characterisation of the Cyp2e1 gene structure

Using the GCG package Best program (Devereux *et al.*, 1984), gapped alignments between the mouse Cyp2e1 cDNA sequence and the human and rat CYP2E1 genes were generated, allowing the prediction of the potential intron and exon boundaries within the Cyp2e1 gene. Based on this data, oligonucleotide primers were constructed to the most 5' and the most 3' regions of the intron/exon junctions for the predicted exons two through to eight and to the most 3' and most 5' region of the junctions for exons one and nine respectively. Using these primers in conjunction with DNA prepared from the three plaque pure λEMBL3 clones hybridising to the 800 base-pair Cyp2e1 cDNA fragment, the structure of the Cyp2c1 gene was assessed using the polymerase chain reaction (PCR). The sizes of the respective introns were illustrated therefore by the sizes of the PCR products generated; the results of these reactions, the predicted intron sizes, the predicted gene structure and the sequences for the oligonucleotides employed are shown in Figures 5.28a and 5.28b.

The results demonstrated that the mouse Cyp2e1 gene has the same basic structure as that seen in the rat and human genes and that the mouse Cyp2e1 coding region is approximately eleven kilobase-pairs in length (Figure 5.28b). From these results also it was possible to quickly assess the portions of the Cyp2e1 gene contained within the λEMBL3 genomic clones. The previously analysed clone and one other clone were seen to contain at least the 3' portion of exon three to the 5' portion of exon nine, and the third clone contained at least the 3' portion of exon one to the 5' portion of exon seven.

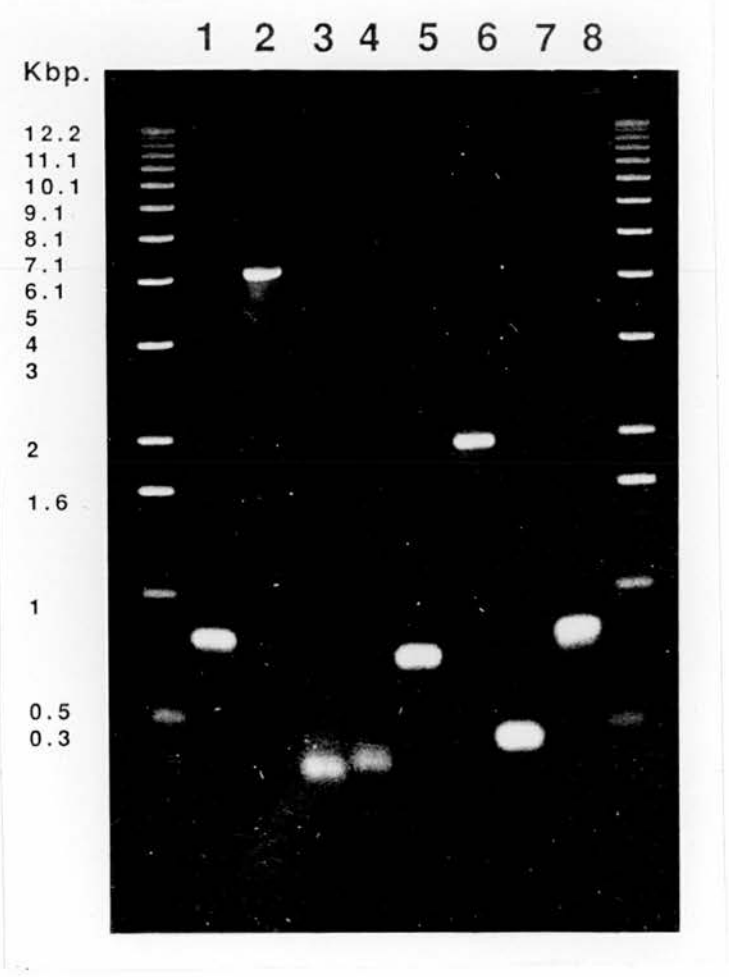


Figure 5.28a: Charaterisation of the Cyp2e1 gene structure. Oligonucleotides to the predicted intron/exon boundaries of the Cyp2e1 gene were used in a PCR mixture to prime DNA isolated from genomic Cyp2e1 clones in λEMBL3. The products of these reactions are shown in conjunction with DNA size markers. Numbers above the lanes indicate introns (e.g. 1 represents the intron between exon 1 and exon 2).

A.

	Size (bp)		
	Human	Rat	Mouse
Exon 1	176	176	176
Intron 1	904	761	800
Exon 2	159	159	159
Intron 2	2938	3404	4100
Exon 3	149	149	149
Intron 3	388	304	250
Exon 4	160	160	160
Intron 4	406	325	300
Exon 5	176	176	176
Intron 5	881	788	750
Exon 6	141	141	141
Intron 6	2836	1873	1900
Exon 7	187	187	187
Intron 7	498	496	450
Exon 8	141	141	141
Intron 8	883	766	850
Exon 9	184	184	184

B.

Region	Exon	Sequence
3′	1	CAGCTGGATTTGAAGGATAT
5′	2	TGGCCCGAAGCGCTTTGCCA
3′	2	TCCTGTGTTCCAGGAGTACA
5′	3	TTCCATGTGGGTCCATTATT
3′	3	CCTGGTGGAGGAGCTCAAAA
5′	4	CCAATCAGAAAGGTAGGGTC
3′	4	TTCTACCTGCTGAGTACTCC
5′	5	TTGTAGATAATCCGAAAAGT
3′	5	GACTGTCTCCTCATAGAGAT
5′	6	CATTGTGTACATGGGTTCTT
3′	6	GATTCTCATGAAATACCCAG
5′	7	ATAACCCTGTCAATTTCTTC
3′	7	GTGTTCCGAGGATATGTCAT
5′	8	AAGGGAGTCCAGAGTTGGAA
3′	8	CAGTGACTATTTCAAGGCGT
5′	9	GCCAGGCCTTCTCCAACACA

Figure 5.28b: Comparing the Cyp2e1 gene structure and those of other CYP2E subfamily members. **A:** A comparison of the known gene structures of the rat and human CYP2E1 genes and the predicted structure of the Cyp2e1 gene **B:** The oligonucleotide sequences to the predicted Cyp2e1 intron/exon boundaries used to characterise the gene structure.

Section 5.8.5: Cloning the 5' portion of the Cyp2e1 gene

In order to assess the potential control functions mediated by the 5' flanking regions of the Cyp2e1 gene, the λEMBL3 clone which was seen to contain at least the 3' portion of exon one was further analysed. Phage DNA was prepared from the recombinant λEMBL clone, subjected to restriction endonuclease digestion with *Sal* I, *Eco*R I, and *Bam*H I, and transferred onto a nylon membrane. The resultant membrane was hybridised the 3' exon 1 oligonucleotide (Figure 5.29). An approximately 4.8 kilobase-pair *Bam*H I fragment, hybridising with the Cyp2e1 3' exon 1 probe was subcloned into the phagemid vector pTZ18 R. The 4.8 kilobase-pair *Bam*H I fragment was characterised for restriction endonuclease sites; using the 3' exon one oligonucleotide as a probe it was seen that exon 1 hybridising portion of the 4.8 kilobase-pair *Bam*H I fragment was localised to a 1.4 kilobase-pair *Bam*H I/ *Hind*III and a similarly sized *Bam*H I/ *Xba*I restriction endonuclease fragment (Figure 5.30). The PCR derived Cyp2e1 intron one (which contains the exon one 3' oligonucleotide sequence) and intron two (which does not contain any exon one sequence) were included on the gel to assist in the washing of the filter. Further restriction endonuclease characterisation of the 1.4 kilobase-pair *Bam*H I/*Hind*III fragment revealed the presence of three *Hind*III sites within the 4.8 kilobase-pair *Bam*H I fragment and not, as may be expected from Figure 5.30, two sites; the *Bam*H I site used in cloning the 4.8 kilobase-pair *Bam*H I fragment has a *Hind*III site very close by and the fragment produced is too small to be seen on an agarose gel or affect the running of the other larger fragments (Figure 5.31). Digestion by the *Hind*III/*Bam*H I restriction endonucleases of the 4.8 kilobase-pair *Bam*H I fragment generated three sub-fragments of 1.1, 1.8 (*Hind*III), and 1.3 (*Hind*III/*Bam*H I) kilobase-pairs respectively; these were subcloned into pTZ18R and pTZ19R phagemid vectors, single-stranded DNA was prepared and portions of the fragments were sequenced. From this analysis it was seen that, on alignment with the known human and rat CYP2E1 gene sequences, the 1.3 kilobase-pair *Hind*III/*Bam*H I fragment contained the upstream region of Cyp2e1, exon one and part of intron one, the 1.8 kilobase-pair *Hind*III fragment contained part of intron one, exon two and part of intron two and the 1.3 kilobase-pair *Hind*III fragment contained part of the large intron two. Double stranded sequencing was also carried out on these fragments using exon oligonucleotides; the sequencing strategy and information gained from this analysis are shown in Figures 5.32, 5.33a, 5.33b, 5.33c and 5.33d. Further analysis of the 5' region of the Cyp2e1 gene and promoter assay studies are discussed in Section 4.11.

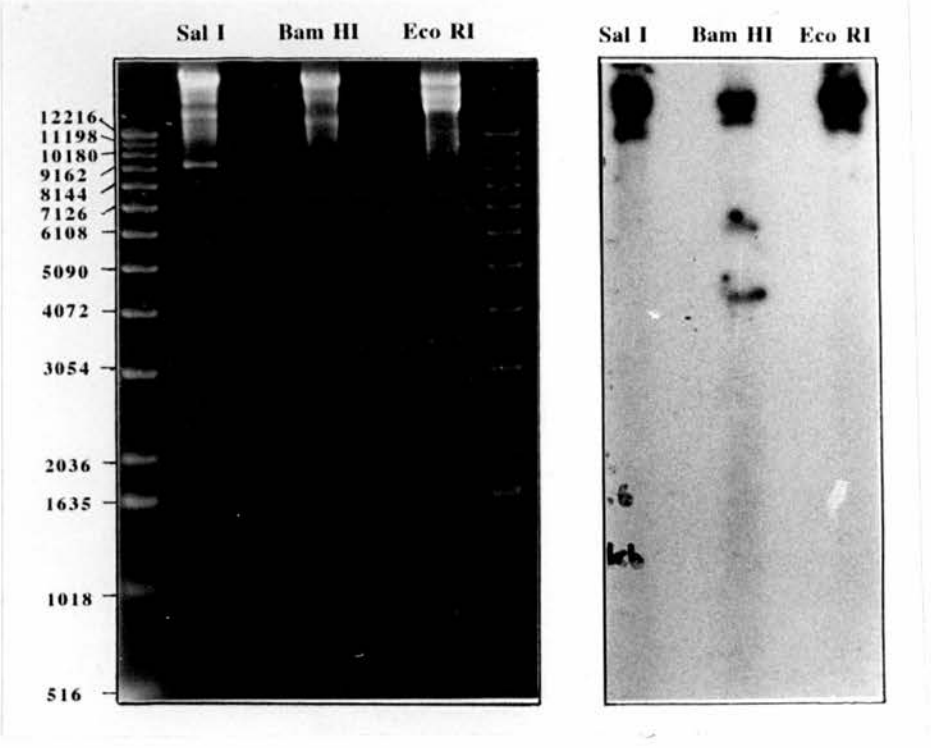


Figure 5.29: Restriction endonuclease analysis of the λEMBL3 clone seen, by PCR analysis, to contain at least the 3 ' region of Cyp2e1 exon 1. The isolated λEMBL3 DNA was digested with the restriction endonuclease indicated, separated on an agarose gel and transferred to a nylon membrane. The membrane was probed using a radioactively labelled oligonucleotide to the 3 ' portion of exon 1. DNA size markers are present and marked sizes are in base-pairs.

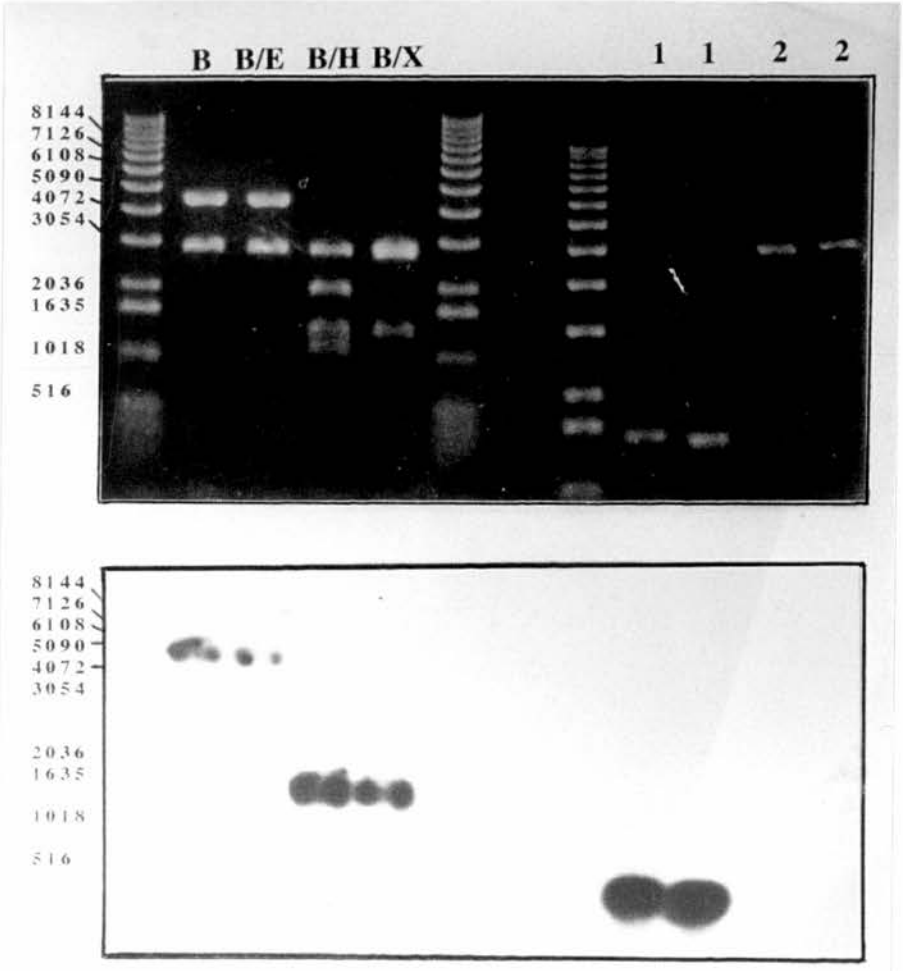


Figure 5.30: Restriction endonuclease analysis of the pTZ18R subcloned 4.8 Kbp *Bam* H I fragment which hybridised to a Cyp2e1 intron 1 oligonucleotide probe. The 4.8 Kbp fragment was subjected to single, or double, restriction endonuclease digestion as indicated **B:** *Bam* H I. **E:** *Eco* R I. **H:** *Hind* III. **X:** *Xba* I. The pTZ18R phagemid vector is seen as a 2.9 Kbp band; the *Bam* H I/ *Xba* I double digest generates a product from the 4.8 Kbp fragment of nearly identical mobility to the vector seen as an intense band at 2.9 Kbp. PCR generated Cyp2e1 gene intron 1 (**1**) and intron 2 (**2**) DNA was also included to aid the generation of specificity in subsequent washing steps. The DNA was transferred to a nylon membrane and hybridised to the radiolabelled Cyp2e1 exon 1, 3 ' oligonucleotide the results of which are seen in the lower box. DNA size markers in base-pairs.

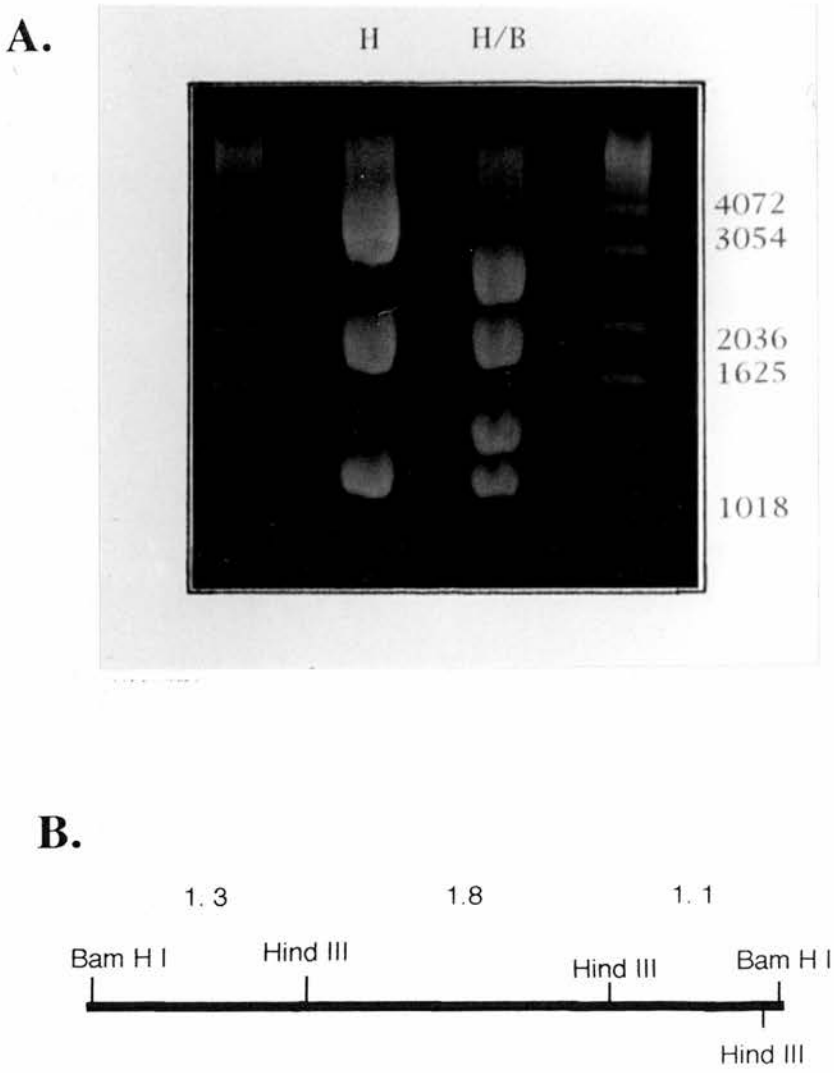


Figure 5.31: Further restriction endonuclease characterisation of the 4.8 Kbp *Bam* H I fragment containing the 5 ' region of Cyp2e1. **A:** The 4.8 Kbp *Bam* H I fragment was digested in reactions with either single or double restriction endonuclease additions as indicated, **H:** *Hind* III, **B:** Bam H I. DNA size markers are in base-pairs and the DNA samples were overloaded as the gel was preparative. This analysis indicated the presence of 3 *Hind* III sites in the *Bam* H I fragment and not 2 as may be expected from analysis in Figure 5.30. **B:** The map of the 4.8 Kbp *Bam* H I fragment indicating the 3 fragments subcloned into the pTZ18 and 19R phagemid vectors. DNA sizes are in K bp.

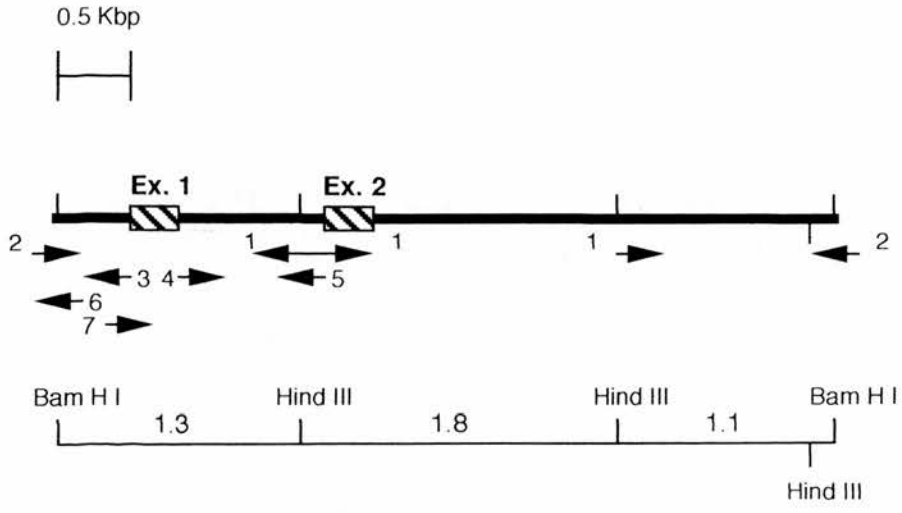


Figure 5.32: The sequencing strategy and oligonucleotides employed to characterise the 4.8 Kbp *Bam* H I fragment containing the 5 ' portion of the Cyp2e1 gene. The *Bam* H I / *Hind* III and 2, *Hind* III /*Hind* III portions of the whole 4.8 Kbp fragment are shown as are the positions and numbers of the exons (**Ex.**); sizes are in Kbp.

Oligonucleotide sequences

1: Reverse sequencing primer
2: Forward sequencing primer
3: TACGGTTCTTGGCGGTA
4: Exon 1, 3 ' (Figure 5.28a)
5: Exon 2, 5 ' (Figure 5.28a)
6: TTAGGAGACCAACGACC
7: TGATAGCCAACTGCAGC

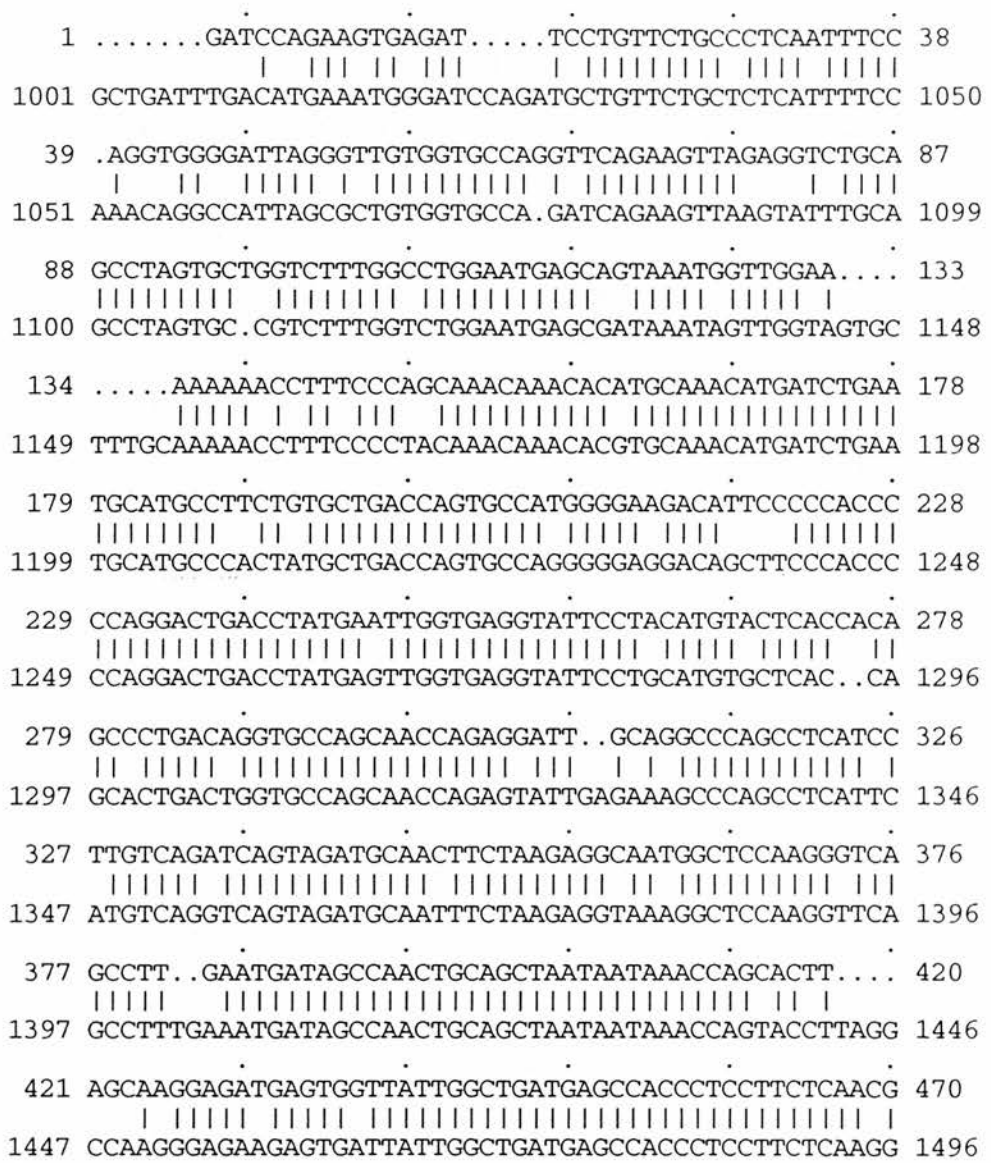


Figure 5.33a: The DNA sequence for the 5 ' portion of the Cyp2e1 gene present in the 1.3 Kbp *Bam* H I / *Hind* III piece of the 4.8 Kbp *Bam* H I fragment. The mouse sequence (upper) was compared to the rat CYP2E1 gene sequence (lower) using the GCG Gap alignment program and the gapped similarity between the two sequences is 81.89%. Exon 1 is boxed; see also Figure 4.15 for analysis of the regulatory elements potentially present within the promoter region of the Cyp2e1 gene.

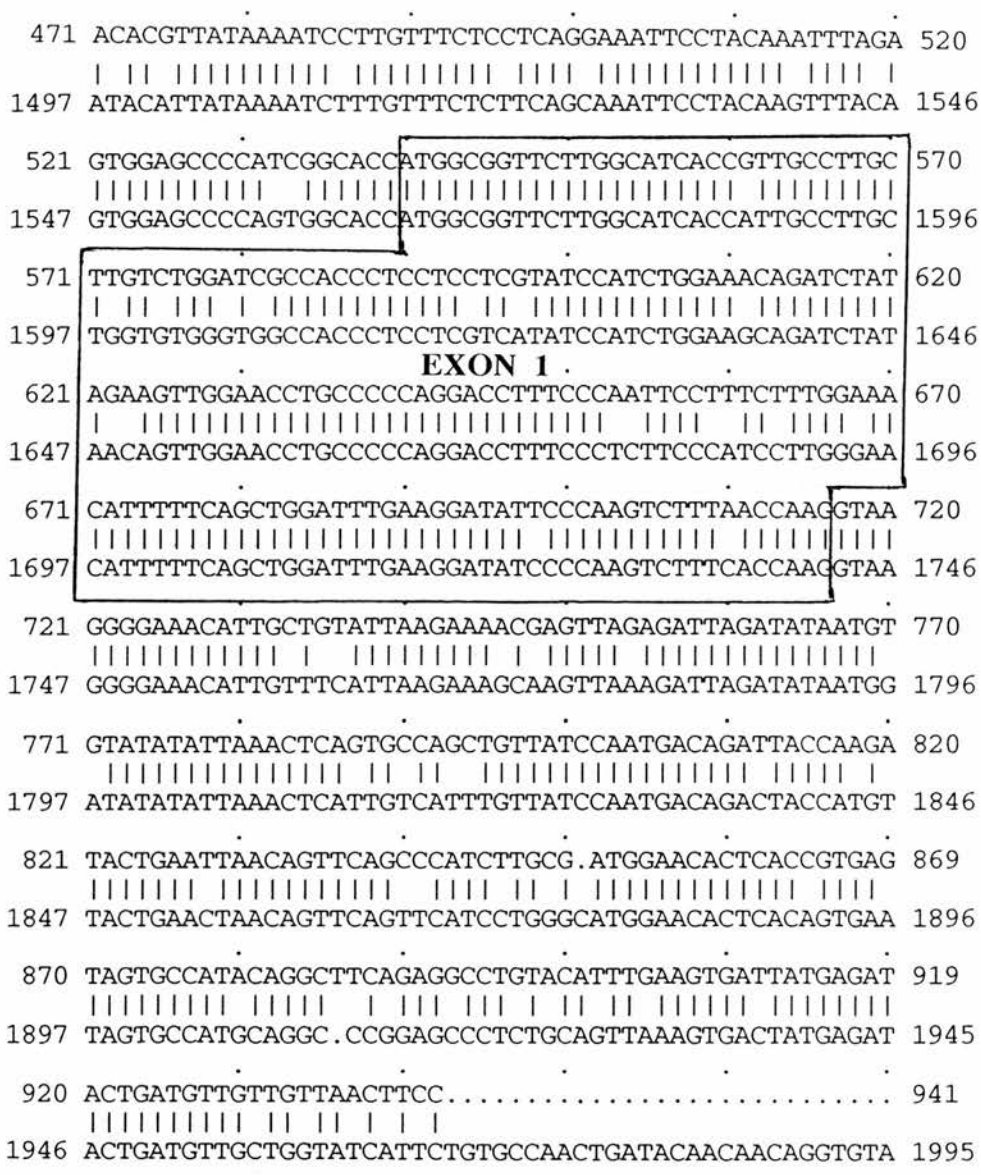


Figure 5.33a (continued)

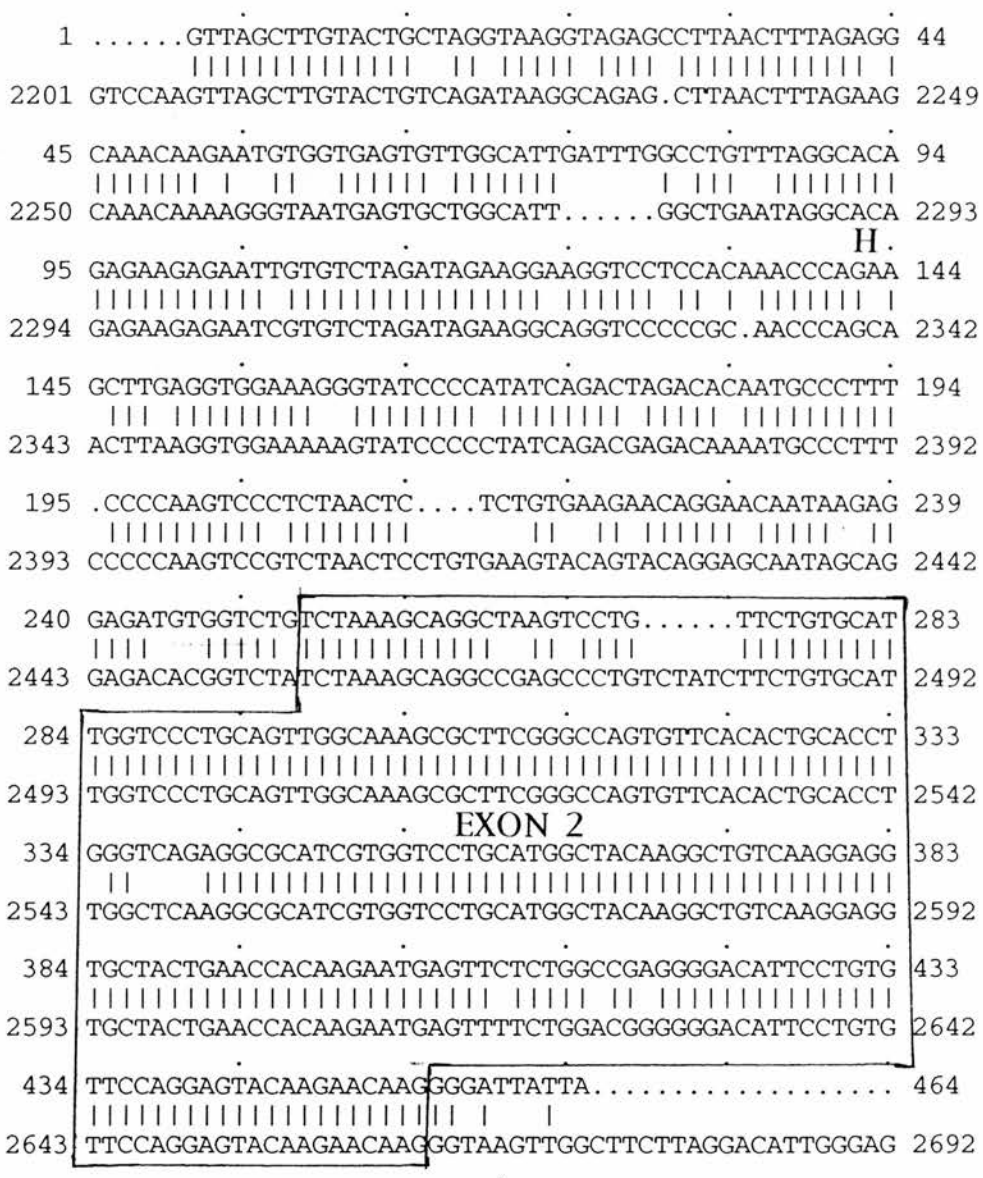


Figure 5.33b: The DNA sequence for the 5 ' portion of the Cyp2e1 gene present in the 1.3 Kbp *Bam* H I / *Hind* III and 1.8 *Hind* III pieces of the 4.8 Kbp*Bam* H I fragment. The mouse sequence (upper) was compared to the rat CYP2E1 gene sequence (lower) using the GCG Gap alignment program and the gapped similarity between the two sequences is 87.5%. Exon 2 is boxed; **H:** Hind III site.

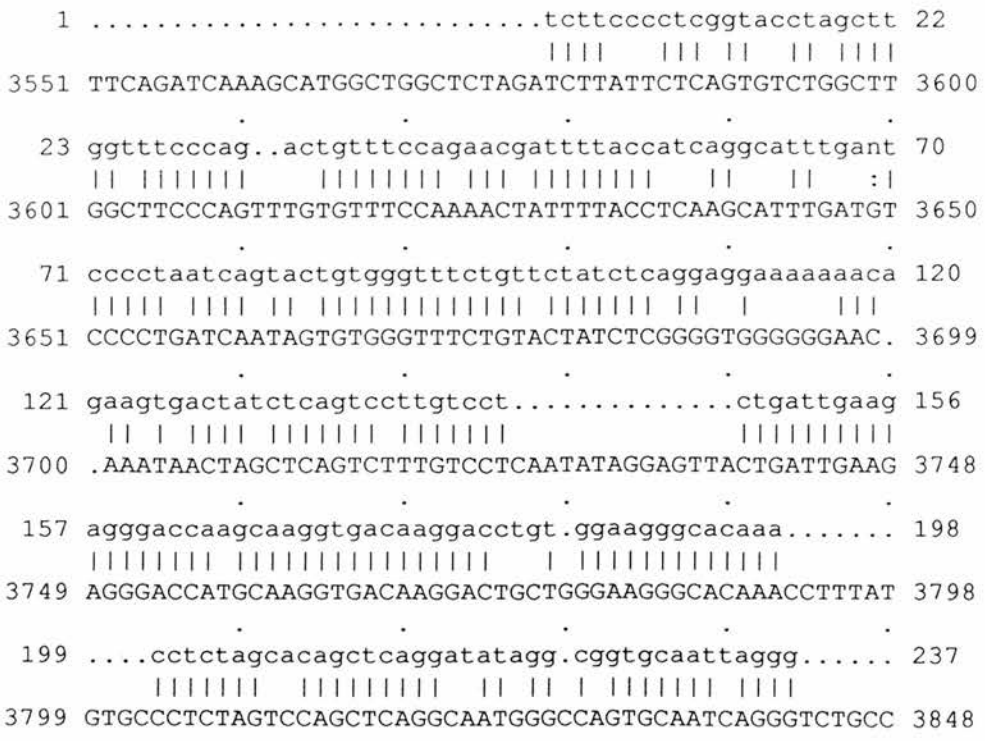


Figure 5.33c: The DNA sequence for the 5 ' portion of the Cyp2e1 gene present in the 1.1 Kbp *Hind* III piece of the 4.8 Kbp *Bam* H I fragment sequenced from the *Hind* III cloning site end. The mouse sequence (upper) was compared to the rat CYP2E1 gene sequence (lower) using the GCG Gap alignment program and the gapped similarity between the two sequences is 78.53%. The sequence corresponds to a portion of the Cyp2e1 gene within intron 2.

Figure 5.33d: The DNA sequence for the 5 ' portion of the Cyp2e1 gene present in the 4.8 Kbp*Bam* H I fragment showing the second *Hind* III (**H**) site of the 1.1 Kbp *Hind* III fragment, close to the*Bam* H I (**B**) original cloning site. The mouse sequence (upper) was compared to the rat CYP2E1 gene sequence (lower) using the GCG Gap alignment program and the gapped similarity between the two sequences is 81.54%. The sequence corresponds to a portion of the Cyp2e1 gene within intron 2.

Section 5.9: Targeting a disruption event in the Cyp2e1 gene

Understanding both the true contribution Cyp2e1 makes to processes such as nitrosamine induced carcinogenesis as well as its possible endogenous gluconeogenic role in the context of the whole organism is very difficult. *In vitro* studies employing, for example, purified Cyp2e1 protein, or Cyp2e1 protein heterologously expressed in bacterial or yeast systems would allow the assay of the mutagenic and metabolic activities of the enzyme in abstraction and only in respect to the particular chemical employed in the assay; the relevance of this data to the *in vivo* role of the enzyme can always be easily questioned.

One potential approach to overcoming the difficulties associated with these methods of analysing the role of Cyp2e1 would be to generate an animal system within which the Cyp2e1 gene had been functionally deleted. Such an approach involves the "targeting" of the Cyp2e1 gene by a homologous piece of DNA, potentially either disrupted or rendered nonsensical, and so generating in turn a non-functional Cyp2e1 gene in the genome as a result of a homologous recombinational event.

Although theoretically possible in any organism, the frequency at which homologous recombination occurs in a particular species varies dramatically. Very high rates of homologous recombination are seen in yeast cells whereas very low rates are experienced in mammalian cell systems. The technique of gene-targeting to date has only been applied in mammalian systems in the mouse, because this is the only species for which pleuripotential cell lines capable of re-integrating into the embryo following manipulation have been established (Evans *et al.*, 1981; Jasin & Berg, 1988). Using this approach it is now possible to generate heritable changes within the genome allowing the potential generation of a mouse with virtually any desired genotype; this approach has been successfully employed on around thirty genes (Capechi, 1990; Frohman & Martin, 1991). Using such an approach it would be potentially possible to study the effect which the loss of the Cyp2e1 gene and its cognate protein would have, not only on the carcinogenic properties of certain chemicals such as the nitrosamines, but also to assess the possible endogenous role of the enzyme through the gluconeogenic behaviour of the animal not expressing the protein in starvation or chemically induced diabetes.

Section 5.9.1: General recombination, the mechanism by which homologous recombination occurs

Although it is possible to introduce very precise changes into the genome of an organism by a process of sequence homology driven gene targeting, the mechanisms by which these processes occur are still not completely understood. General recombination within eucaryotic cells, that is recombination dependent on the sequence homology between two sequences and not on specific sequence motifs generating recombination, occurs at meiosis, mitosis, and during DNA repair. The result of this general recombination can either be the reciprocal exchange of two double-stranded molecules,"crossing-over", or a non-reciprocal process of "gene-conversion", in which information is passed from one duplex to another in one direction only.

Most of the information on these general recombination processes has been gained from studies in yeast and other fungi and the reactions have a common theme in which a free end of single-strand of DNA invades a homologous region of double stranded DNA to produce a joint molecule. This intermediate joint molecule passes through various intermediates leading to cross-exchanges of DNA which finally resolves, resulting in various permutations of DNA exchange (Holliday, 1964). Similar processes have now been shown to occur as the result of double-strand initiated breaks and invasions of homologous DNA stretches (Orr-Weaver *et al.*, 1981). The enzymology and relative importance of the two general recombination patterns *in vivo* in yeast systems are not well understood.

The enzymology of recombination in bacteria, principally *E. coli* , has been more fully elucidated however and parallels have been drawn between these processes and those observed in yeast and other eucaryotic cells (Smith, 1989).

Studies in mammalian cell culture systems have provided information on the mechanisms of recombination in higher eucaryotes. Extrachromosomal recombination has been studied in tissue culture systems by assaying for the regeneration of selectable markers. For example recombination can be assayed by the generation of a functional neomycin resistance gene as a result of homologous recombination between two non-functional neomycin resistance genes mutated at different sites; subsequent selection of the cells then shows the numbers in which homologous recombination has occurred (Bollag *et al.*, 1989). These studies have shown that there is measurable homologous recombination with as few as twenty-five base-pairs of homology between sequences and that the frequency of recombination increases as the extent of the homology rises. The increased frequency of recombination with increased DNA fragment size is however biphasic suggesting that at least two recombinational mechanisms are operating in mammalian cells with one operating predominantly on DNA fragments

below 150-400 base-pairs in length and one above this size transistion (Rubnitz & Subramani, 1984; Ayares *et al.*, 1986). The introduction of a double-strand DNA break in the sequences used in these studies leads to an increase in the frequency of recombination as does the inclusion of a transcriptionally active piece of DNA into the target fragment. These results suggest that there is a role for the opening of the DNA duplex, or relaxation of the chromatin environment, in the establishment of the recombinational processes (Nickoloff & Reynolds, 1990).

Inter-chromasomal recombination in mammalian cells has been studied using a similar approach to that used to analyse extra-chromosomal recombination. The introduction of selectable markers containing various complementable mutations into the genome of tissue culture cell lines allows the selection of cell lines in which a productive homologous recombinational event has occurred. The frequency of homologous-recombination observed in these experiments is lower than that obtained in similar studies on extra-chromosomal recombination. However, as observed in extra-chromosomal recombination studies there is a increased frequency of recombination as the fragment size increases with a requirement of between 134 and 232 base-pairs of homology before any recombinational events are detected (Bollag*et al.*, 1989; Liskay *et al.*, 1987; Waldman & Liskay, 1988). The introduction of non-homologous sequences into the region of homology reduces the level of homologous recombination suggesting that above a certain size non-homologous stretches of DNA may effect the stability of the recombining hetero-duplex (Waldman & Liskay, 1988; Letsou & Liskay, 1987).

As well as homologous recombination, non-homologous recombination also occurs in mammalian cells and this process is predominant; integration of non-homologous DNA is thought to occur at the site of a chromosomal break and the levels of this type of recombination are seen to be elevated on the generation of double-stranded DNA breaks following treatment of the cells with ionising or DNA damaging chemicals (Perez *et al.*, 1985). The level of non-homologous recombination is in great excess of the level of homologous recombination in mammalian systems. Overlaps between homologous and non-homologous recombination processes have been proposed with common proteins, such as the homologous pairing protein (HPP-1), for example, being capable of catalysing either event (Fishel *et al.*, 1991).

Section 5.9.2: Gene targeting

By fusing the approaches used to study the processes of intra- and extra-chromosomal recombination, the possibility of gene targeting was first investigated. The first experiments using this technique studied the possibility of regenerating a selectable gene marker by homologous recombination between a non-functional genomically integrated marker and a complementing non-functional, non-replicative homologous extra-chromosomal marker. These experiments gave ratios of non-homologous targeting frequencies to homologous targeting frequencies in the range of 10^{-2} to 10^{-7} and integration frequencies of any type in the region of 10^{-3} to 10^{-7}. Differences in both the frequency of integration *per se* and the ratio of homologous to non-homologous recombination were obtained depending on the cell type and the nature of the DNA target employed. These differences presumably related to variations in the background levels of general recombination present in the different cell types and the different chromosomal locations of the various target sequences (Smith & Berg, 1984; Lin *et al.*, 1985). It was seen that the rates of homologous recombination did not increase, unlike the frequency of random integration, with an increase in the amount of exogenously added DNA, suggesting that the location of the homologous stretch of DNA in the genome was not the rate limiting step (Thomas *et al.*, 1986). Many studies on the generation of homologous recombinational events within endogenous genes exploited the use of the selectable hypoxanthine-guanine phosphoribosyl transferase gene (HPRT) as a target locus. Disruption of the HRPT gene generated resistance to the base analogue 6-thioguanine and so no other external selection system needed to be applied; the HPRT gene also had the advantage of being on the X chromosome and so deletion of one copy was all that was required to completely remove the gene from the male genome. Deficiency of HPRT in humans is associated with severe mental retardation which made it a prime candidate for pioneering medical gene replacement studies. These cell culture studies showed that targeting frequency was accelerated by the presence of double-stranded DNA breaks in the region of homology but that the presence of large pieces of non-homologous DNA, up to 2.5 kilobase pairs, did not substantially reduce the homologous targeting frequency (Valencius & Smithies, 1991).

Other studies targeted endogenous genes without an internal selection by interupting the gene with a selectable marker. The first endogenous mammalian gene to be modified by this approach was the β-globin gene, the targeting vector contained the neomycin resistance gene to act as a positively selectable marker for recombination. The ratio of homologous to non-homologous recombination was 1:1000 yet it was also observed that the same frequency of homologous recombination was observed in cell lines expressing the gene, as those that were not (Smithies *et al.*, 1985).

Vectors employed in these experiments could either generate a replacement event ("replacement vectors") with a marker interrupting the homologous piece of DNA, or they could generate an insertion event ("insertion vectors") where a marker sequence is placed outside the homology seeking piece of DNA, requiring therefore a double cross-over event to occur in order to allow the disruption of the target gene. Both these vector types were seen to operate with a similar frequency of homologous targeting, leading to the suggestion that targeting operates not via initiation of a homologous DNA exchange duplex at one site rather by following two separate cross-over events (Thomas & Capechi, 1987; Ellis & Bernstein, 1989).

In order to allow an increase in the ease of selection of homologous as opposed non-homologous recombination events, a new type of targeting vector and method of selection, termed "positive-negative" selection (PNS), were developed. These vectors allowed the use of a positive selection marker, such as neomycin resistance, to demonstrate that recombination had taken place, and also a negative selection marker to select against the higher frequency of non-homologous recombination events (Mansour *et al.*, 1988). The negative selection is obtained by the incorporation of a selectable marker such as the Herpes-simplex viral thymidine kinase gene (HSVTK) which, if integrated into the genome of the recipient cell line, will render the cell sensitive to the base analogue gancyclovir. This gene is placed outside the region of homologous DNA being used to targeted the construct and so should be lost on resolution of homologous recombination. In the case of a non-homologous recombination event however, the HSVTK gene would be retained and integrated into the genome in conjunction with the rest of the targeting vector and so non-homologous integration can be selected against. The positively selectable marker is placed within the region of homologous DNA being targeted and so is not necessarily lost in the event of either a correctly or incorrectly targeted recombinational event. Thus the correct homologous recombinational event between the incoming targeting vector and the host genome can be selected for by the ability of the cell to withstand positive selection of neomycin, or G418, whereas the non-homologous recombinational event can be selected against by the use of negative selection by gancyclovir (Mansour *et al.*, 1988). This selection system is summarised in Figure 5.34.

In order to allow the regeneration of a complete mouse following a correctly targeted homologous recombination event, the targeting and selection have to be carried out in specific mouse cell lines capable of subsequently contributing to a chimeric mouse germ line. Such a cell line, termed the "Embryonic Stem" cell line (ES), has been established from a mouse embyro-derived teratocarcinoma; the ES cells are pluripotent and capable therefore of contributing to the germ-line of a mouse if reintroduced, following targeting manipulations, to the blastocyst (Evans *et al.*, 1980; Bradley *et al.*, 1987).

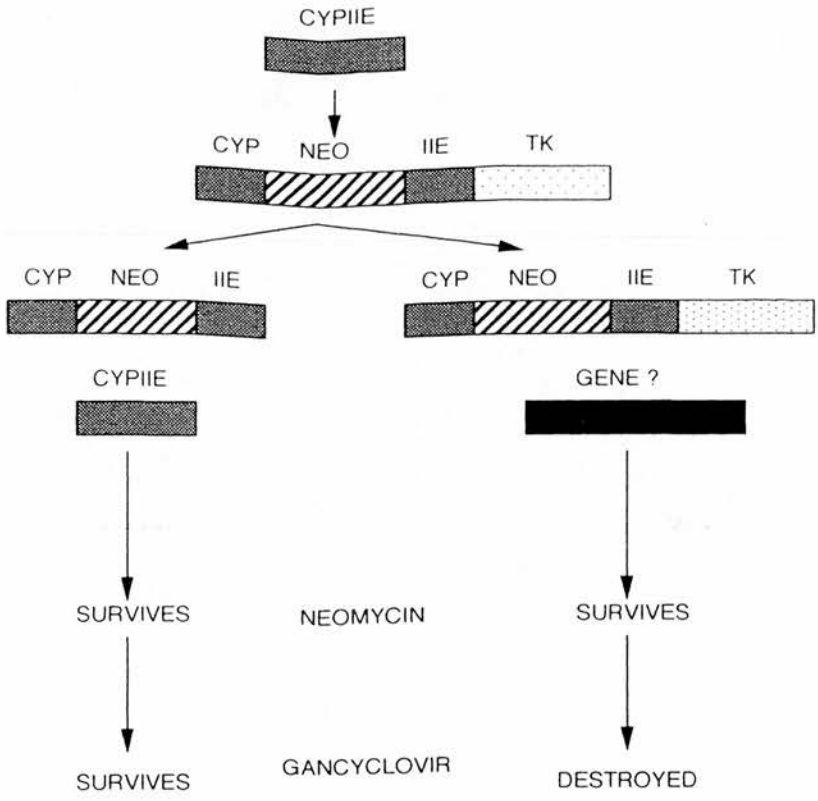


Figure 5.34: The positive-negative selection strategy employed to target a disruption event in the Cyp2e1 gene. Theoretically if the construct homologously recombines with the Cyp2e1 gene in the genome the neomycin (**NEO**) gene should disrupt the Cyp2e1 gene but the thymidine kinase gene (**TK**) should be lost as illustrated on the left. The combinaton of these events should generate a cell resistant to neomycin but not sensitive to gancyclovir. Conversely, as illustrated on the right, if the construct integrates randomly into the genome although the cell is resistant to neomycin it is sensitive to gancyclovir selection.

Section 5.9.3: Constructing a Cyp2e1 positive-negative insertional gene targeting vector

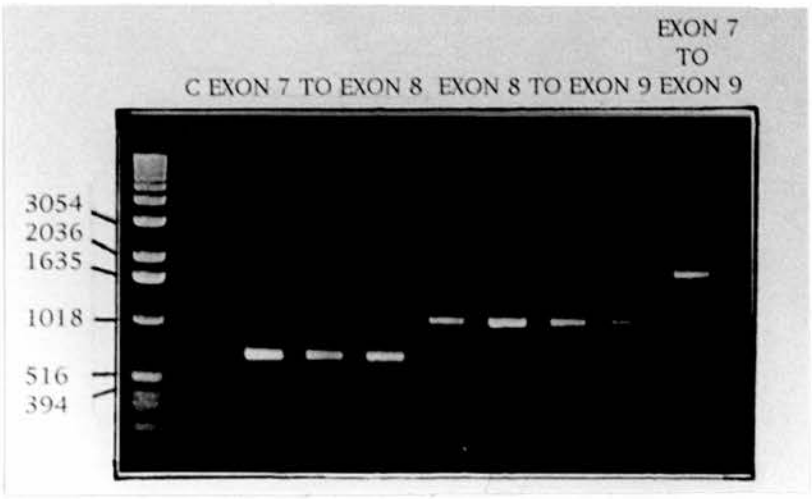
In order to attempt to generate a mouse line which did not possess a functional Cyp2e1 gene, a positive-negative insertional gene targeting vector containing the 3' portion of the Cyp2e1 gene was constructed. Cyp2e1 is unique in that it is a single copy gene unlike many of the other subfamilies in the P-450 family 2 which have expanded presumably as a result of gene duplication events. Cyp2e1 is therefore a feasible target for gene deletion with the possibility of removing its enzymatic activity from the mouse without the complication of closely related gene products phenotypically masking the consequence of its loss.

The positive-negative (PNS) vector employed contained a positively selectable neomycin gene (Stratagene), a yeast *leu 2* selectable marker, and a negatively selectable HSVTK gene on a backbone derived from the plasmid pBS and was the kind gift of Drs D. Porteous and A. Brooks, MRC Human Genetics Unit, Edinburgh; collectively this plasmid was termed pHRPNS$_{TK}$. The yeast *leu 2* selectable marker gene was incorporated into the vector to allow the possibility of the use of the vector in the generation of yeast artificial chromosomes (YACs). A vector was constructed using sequences from the 3' end of the Cyp2e1 gene which had previously been cloned (Section 5.8.3). The 3' region of the Cyp2e1 gene was chosen as it encodes a region of the Cyp2e1 protein that, on alignment with the known structure of the bacterial P-450cam, contributes to the binding of the haem prosthetic group (Section 5.3.5). Therefore, disrupting the protein around this area, even if a truncated protein was generated, would lead to the production of a non-haem containing Cyp2e1 apoprotein which would not be capable of catalysing any of the reactions normally undertaken by Cyp2e1. Portions of the Cyp2e1 gene downstream of exon 9 are not suitable for incorporation into a PNS vector; inclusion of the β2 SINE present in the 3' UTR of the Cyp2e1 gene in the PNS vector would complicate the selection system as it would potentially lead to the insertion of the targeting sequence at any of the several thousand β2 SINE sequences scattered throughout the genome (Section 5.4.1). The restriction endonuclease sites present within the pHRPNS$_{TK}$ vector did not correspond to any that were naturally available within the 3' fragment of the Cyp2e1 gene and so the pieces to be used in the construct were generated by the polymerase chain reaction (PCR). In this manner, two Cyp2e1 derived PCR fragments, one containing exon 7, intron 7, and a 5' portion of exon 8 (around 600 base-pairs in length) with *Xba*I and *Sal*I restriction endonuclease sites, and the other containing a 3 ' portion of exon 8, intron 8, and exon 9 (around 1000 base-pairs in length) with *Bam*H I restriction endonuclease sites were generated (Figure 5.35). The regions of the Cyp2e1 gene spanned by these fragments

contained a 50 base pairs deletion in exon 8 which effectively truncated the Cyp2e1 protein at leu 396 and led to the loss of the highly conserved pocket around the haem binding cys 435 (Section 5.3.2). The fragments were subcloned into pTZ18 R and pTZ19 R phagmids and sequenced confirming them to be the expected regions of the Cyp2e1 gene. The fragments were initially subcloned into the phagemid vectors in this manner to ensure that the PNS vector contained clonal fragments and also to exploit the α-complementation selection system (Section 2.11.7a) available in the phagemids but not possible in the $pHRPNS_{TK}$ vector.

The exon 7, intron 7 and 5' portion of exon 8, *Xba*I/*Sal*I fragment was then directionally subcloned from the phagemid into the *Xba*I and *Sal*I restriction endonuclease sites of the $pHRPNS_{TK}$ vector; recombinant colonies were selected by colony hybridisation using the subcloned exon 7, intron 7 and 5' portion of exon 8 as a probe. This leads to the 5' portion of exon 8 being interupted by the neomycin resistance gene. The 3' exon 8 portion, intron 8 and exon 9 *Bam*H I restriction endonuclease site fragment was then subcloned into the exon 7, intron 7 and 5' portion of exon 8 containing $pHRPNS_{TK}$ construct and recombinant clones selected by colony hybridisation using the subcloned 3' exon 8, intron 8 and exon 9 fragment as a probe. Clones which contained the correctly orientated exon 8, intron 8 and exon 9 *Bam*H I restriction endonuclease site fragment were then assessed through the use of PCR in conjunction with the oligonucleotides originally employed to generate the Cyp2e1 fragments. If the oligonucleotides to the 5' of exon 8 and the 3' of exon 9 were employed in the reaction, only those clones which contained the correctly orientated *Bam*H I fragment, that is with the exon 8 portion adjacent to the neomycin resistance gene, would generate a product, which now will also contain the neomycin resistance and *leu 2* genes. The completed correctly orientated vector was termed $pHRPNS_{TK}$ Cyp2e1; this was then digested with *Xba*I/*Sal*I , and *Bam*H I restriction endonucleases separately and as a triple digest to release the various Cyp2e1 components and analysed by Southern blotting, and probed with the two Cyp2e1 fragments used in the construction of $pHRPNS_{TK}$ Cyp2e1 (Figure 5.36).

A.



B.

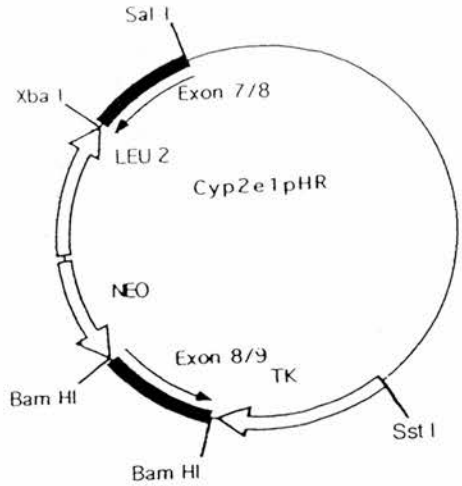


Figure 5.35: The generation of the pHRPNS$_{TK}$Cyp2e1 construct. **A:** The portions of the Cyp2e1 gene amplified from the 3 ' *Bam* H I Cyp2e1 genomic clone, annotated as follows: **c:** Control reaction with no DNA template in the reaction mixture, the 7 other tracks are as indicated and contain the portions of the Cyp2e1 gene used in the pHRPNS$_{TK}$Cyp2e1 construct. The final track represents the complete region of the Cyp2e1 region amplified including all of exon 8 and was not employed in the construct. Oligonucleotides

	Restriction site	Region annealing to Cyp2e1 exons
5 ' Exon 7:	TCGAATCTAGA	GAAACTTCATGAAGAAATTGACAGGGTTATT
5 ' Exon 8:	TCGAAGTCGAC	GGAGTCCAGAGTTGGAATCACAACTGTACC
3 ' Exon 8:	TCGAAGGATCC	GCATTTCCTGAATGAAAATGGGAAGTTCAA
3 ' Exon 9:	TCGAAGGATCC	GGGATACTGCCAAAGCCAACTGTGACAGG

B: A diagram of the final pHRPNS$_{TK}$Cyp2e1 construct illustrating the restriction sites relevant to the generation of the pHRPNS$_{TK}$Cyp2e1construct and its linearisation prior to electroporation into ES cells (**TK:** thymidine kinase gene. **NEO:** neomycin resistance gene). The diagram is not to scale and the arrows above the Cyp2e1 fragments indicate the direction of transcription in the native gene.

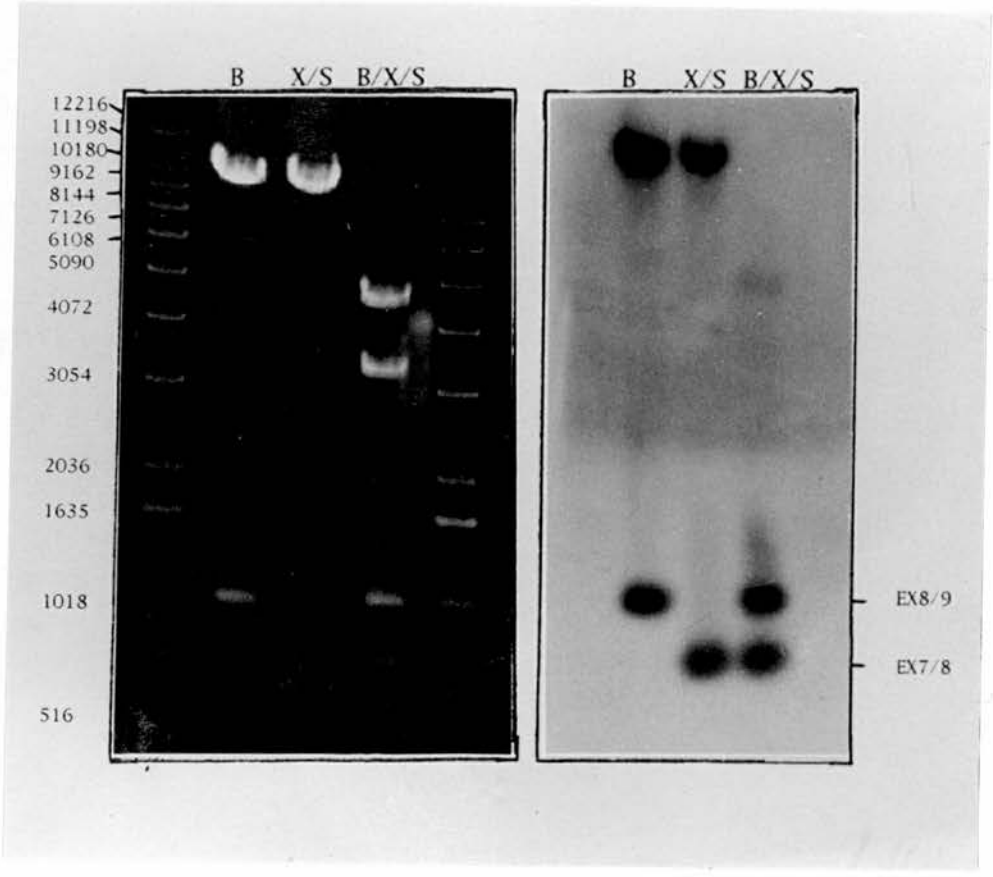


Figure 5.36: Restriction endonuclease confirmation of the final pHRPNS$_{TK}$Cyp2e1 construct. The completed pHRPNS$_{TK}$Cyp2e1 construct was subjected to single, double or triple restriction endonuclease digestion as indicated (**B:** *Bam* H I. **X:** *Xba* I. **S:** *Sal* I). The construct generated the expected DNA fragment pattern following separation on an agarose gel (See Figure 5.35) and was transferred to a nylon membrane.The membrane was probed with radiolabelled DNA fragments corresponding to exon 7 to 8 (**EX 7/8**) and exon 8 to 9 (**EX 8/9**) of the Cyp2e1 gene (the fragments of the Cyp2e1 gene present in the pHRPNS$_{TK}$Cyp2e1 construct) and annealed to the expected DNA fragments.

Section 5.9.4: Transfection and positive negative selection of the pHRPNS$_{TK}$ Cyp2e1 construct in Embryonic Stem (ES) cells

DNA preparations of the pHRPNS$_{TK}$ Cyp2e1 vector were digested with the restriction endonucleases *Xba*I and *Sst*I to release the neomycin resistance gene interupted Cyp2e1 gene fragment and the flanking HSVTK gene (Figure 5.35). The DNA was then electroporated into ES cells. Positive-negative selection was applied and 16 clones survived both the positive, neomycin, and negative, gancyclovir, selection; genomic DNA was isolated from these clones (Section 2.23.8). These manipulations were performed in colaboration with Dr J. Dorin, MRC Human Genetics Unit, Western General Hospital, Edinburgh.

Section 5.9.5: Screening for correctly targeted pNS$_{TK}$Cyp2e1 in the ES cell line

The genomic DNA isolated from the sixteen ES clones which survived both the positive and negative selection steps were analysed to see if any had arisen from a correctly targeted homologous recombination event using a PCR assay. A PCR assay was used for the screening of the clones due to the limited amount of genomic DNA available after the selection process and the relatively small fragment sizes of the Cyp2e1 gene which needed to be amplified; such an approach has led to the successful detection of gene targeting in several studies (Kim *et al.*, 1988; Zimer & Grus, 1989; Joyner *et al.*, 1989; Smithies *et al.*, 1989; Sanona *et al.*, 1991). Optimal PCR conditions were assessed for the spooled DNA samples obtained from the ES cell clones using oligonucleotide primers which anneal within intron 6 and exon 8 of the Cyp2e1 gene. These oligonucleotides generate an approximately 650 base-pair fragment resulting from the amplification of portions of the Cyp2e1 gene both inside and outside the areas used in the generation of the pHRPNS$_{TK}$ Cyp2e1 construct. When the best conditions for the reaction had been obtained (Section 2.10.2), the exon 8 primer was replaced with an oligonucleotide primer which annealed to the 3' region of the yeast *leu 2* gene. Only DNA from the clones in which the pHRPNS$_{TK}$ Cyp2e1 construct had correctly targeted the Cyp2e1 gene would generate a PCR product from this primer set, as in the normal mouse genome such a juxtapositioning of sequences does not occur. No products however were generated from these or several variations of the oligonucleotide primer sets both to the *leu 2* and neomycin resistance genes and other regions of intron seven. Using oligonucleotide primers to the 5' coding region of the neomycin gene and the *leu 2* gene it was seen that PCR products were generated suggesting that the pHRPNS$_{TK}$ Cyp2e1 construct had somehow inserted non-homologously into the genome of the surviving ES cell clones; these products

were larger than expected and this observation suggested that modification and concatermerisation of the construct had occurred, either during or following integration into the genome. The fact that a product was generated by the intron 7 and exon 8 oligonucleotide pair suggests that the failure of the intron 7 and *Leu 2* oligonucleotide pair to do so was not due to an inability to amplify this portion of the genome and it is more likely that no correctly targeted recombinational event had occurred (Figure 5.37).

Section 5.9.6: Possible reasons for the non-targeted pHRPNS$_{TK}$ Cyp2e1 construct integrations and future improvements to the Cyp2e1 deletion system

Although employing a PNS system in using the pHRPNS$_{TK}$ Cyp2e1 construct theoretically should increase the frequency of homologous recombination events, several observations as to why non-homologous integration of the construct may have occurred have been noted.

In several studies the HSVTK gene was seen to become mutated before or after random integration making the cells in which this has occurred resistant to gancyclovir and appear phenotypically to have undergone a homologously integration event although the HSVTK portion of the gene is not physically lost. It has been suggested that the inclusion of two HSVTK genes into the constructs may reduce the problem associated with the selection of a mutated integrated from of the construct (Mansour*et al.*, 1988).

A marker variation in the frequency of correct targeting events occurred depending on the nature of the target locus; the correct targeting of the *int-2* gene for example was seen to be around twenty-fold lower than that for the HPRT gene using effectively similar constructs. It was suggested that this difference in targeting frequency related to the relative transcriptional levels of the target genes; *int-2* is expressed at very low levels, whereas the HRPT gene expression is relatively high in ES cells which may produce an effect on the nature of the chromatin surrounding the target locus. Potentially if the target locus is not expressed the chromatin associated with it may be highly compacted either lowering the ability of the recombinational machinery to locate and initiate the exchange between the incoming construct DNA and the genome, or the ability of the transcriptional machinery to generate transcripts from either the positive or negative selection markers (Mansour, 1988). Cyp2e1 message could not be detected in mouse embryonic RNA (kind gift, Dr. R. Hill, MRC Human Genetics Unit, Edinburgh) using the 800 base-pair Cyp2e1 partial cDNA as a probe, and studies on the CYP2E1 gene in other species suggest that the gene is not transcriptionally active prior to birth (Umeno *et al.*, 1988; Bonfils *et al.*, 1990; Jones *et al.*, 1992).

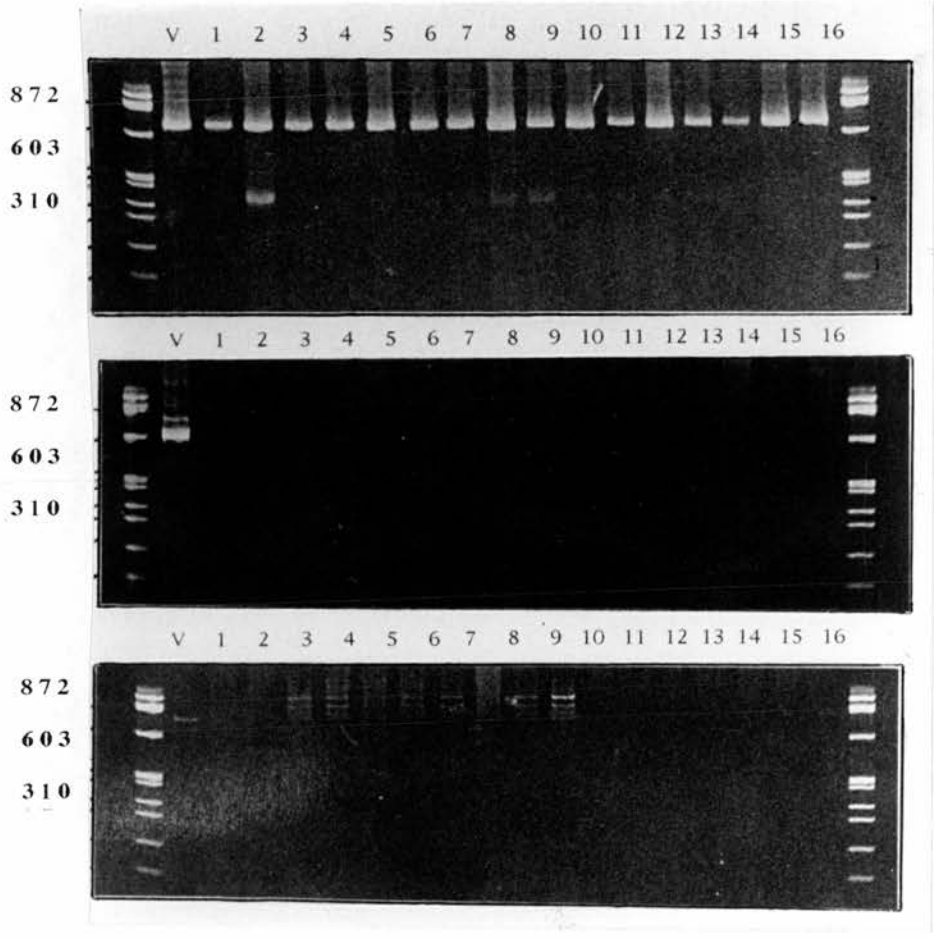


Figure 5.37: Screening the 16 surviving ES cell clones for a gene targeting event in the Cyp2e1 gene. **A:** The conditions for the PCR were established using oligonucleotides to intron 6 and exon 8. **B:** The exon 8 oligonucleotide was replaced with a *Leu 2* 3 ' oligonucleotide; the intron 6 oligonucleotide from the previous reaction (A) was retained. **C:** The intron 6 oligonucleotide was replaced by an internal oligonucleotide to the neomycin resistance gene; the *Leu 2* oligonucleotide from the previous reaction (B) was retained. Annotation: The 16 (**1-16**)ES samples are indicated **V:** The pHRPNS$_{TK}$Cyp2e1 construct was included as a contol for the PCR. DNA marker sizes are indicated in base-pairs.

Oligonucleotides:

Intron 6*: TTATCCCTATCTTGGCA Exon 8: Exon 8, 5 ' (Figure 5.28b)
Leu 2*: TCTATAAAGAACTTCGT Neo*: GGCAGCACCGGTCGGTG

*Oligonucleotides were obtained, and assays performed, for both DNA strands.

These observations suggest that the Cyp2e1 gene may not be transcriptionally active in the ES cells and so the surrounding chromatin structure may not be conducive to the generation of a recombination event. These observations relate directly to results gained from studies of extra-chromosomal recombination where higher recombination frequencies were noted if transcriptionally active portions of DNA were included in the recombining fragments (Nickoloff & Reynolds, 1990). In contrast to these observations, however, it was noted that the frequency of targeting of the β-globin locus was independent of whether the cell type expressed the gene or not (Smithies *et al.*, 1985; Johnson *et al.*, 1989).

Other reasons for the lack of a correctly targeted recombination event may lie in the construction and nature of pHRPNS$_{TK}$ Cyp2e1 itself. In a series of experiments studying the frequency of homologous targeting of the HRPT gene in ES cells several inportant factors regarding the nature of the targeting construct have been identified. Studies viewed the effects of the length of the homologous DNA used in the construct and the level of sequence homology between the incoming extraneous construct DNA compared with the genomic target DNA (Deng & Capecchi, 1992). The length of the homologous DNA used in the construct strongly affects the recombination frequency obtained; it was seen that 2 kilobase pairs of homologous DNA, similar in size to the amount of homologous Cyp2e1 sequence used in the pHRPNS$_{TK}$ Cyp2e1 construct, produced a targeting frequency ratio of non-homologous to homologous recombination between 10^{-6} to 10^{-7} whereas if the length of the targeting sequence is increased, the efficiency increased up to a ceiling limit of around 14 kilobase-pairs, where the recombination system appears to saturate at a recombination frequency of around 10^{-5}. Although in these experiments the smallest homologous fragment size used was around two kilobase-pairs, other studies have successfully employed fragment sizes of less than a single kilobase (Hasty *et al.*, 1991). It is also apparent that extremely small amounts of DNA can be handled by the homologous recombination system leading to the generation of clusters of SINEs in areas of the genome, for example in the rat growth hormone gene (Barta *et al.*, 1981) or a human β-tubulin gene (Lee *et al.*, 1984). Therefore, in the case of the rodent β2 SINE family, the recombination system if the same mechanism is employed in this instance as in other cases of general recombination, is capable of homologously recombining DNA fragments as small as 150 base-pairs in length (Section 5.4.2). In intra-chromosomal recombination it was suggested that two different recombination systems may operate with one recognising smaller fragments (150 to 400 base-pairs of homology) and a different system recognising fragments above this size limit ((Rubnitz & Subramani, 1984; Ayares *et al.*, 1986).

The source of the DNA used to generate the construct is now seen to play an important function in the level of homologous recombination achieved. If "isogenic" DNA, that is genomic DNA from the same mouse line as that from which the ES cells were derived (C129), as opposed to non-isogenic DNA is used in the construction of the targeting construct then around a twenty-fold increase in targeting efficiency is observed. The base differences between the HPRT gene in the CC1.2 genome and the BALB/c and Swiss-Webster mice lines were only around 1% different yet still this led, at a targeting fragment size of two kilobase-pairs, to a shift in the efficiency of homologous recombination frequency from 10^{-6} (isogenic) to nearer 10^{-7} (Deng & Capacchi, 1992); similar levels of increased recombinational efficiency were also reported for the targeting of the retinoblastoma gene by isogenic as opposed to non-isogenic DNA fragments (teReile *et al.*, 1992). It is seen in bacterial recombination that the process of exchange of DNA between two homologous regions is edited by a mismatch repair enzyme system which leads to the termination of the crossing-over event if too many mismatches are detected between the two sequence fragments (Rassiguier *et al.*, 1989; Shen & Huang, 1989); it is possible that a similar system may be operating in mammalian homologous recombinational processes. The presence of stretches of totally non-homologous DNA adjacent to the targeting sequence however does not appear to greatly effect the targeting efficiency, suggesting that a mismatch system may operate only when the DNA fragments in question show a certain level of homology (Mansour *et al.*, 1988).

It is possible that strain differences between the BALB/c derived Cyp2e1 fragment used in the $pHRPNS_{TK}$ Cyp2e1 construct and that portion of the Cyp2e1 gene in the C129 genome may contain sequence divergence particularly within the intronic regions not so highly conserved by coding restraints. This may lead to a decrease in homology levels between the ES Cyp2e1 gene and those portions present on the targeting construct.

Studies have shown that the frequency of homologous recombination is not affected by the presence of as much as 12 kilobase-pairs of adjacent non-homologous DNA (Mansour *et al.*, 1988). These observations suggest that the large pieces of non-homologous DNA may become looped out from the homologous hetero-duplex during recombination; studies in this area in intra-chromosomal recombination however have suggested that the insertion of large stretches of non-homologous DNA may reduce the frequency of recombination (Letson & Liskay, 1987). It is not clear therefore whether the effect of the inclusion of the non-essential *leu 2* gene in the $pHRPNS_{TK}$ Cyp2e1 construct as well as the neomycin resistance gene would be expected to have any effect on the potential targeting efficiency of the construct.

Section 5.9.7: The generation of a more efficient Cyp2e1 targeting construct

It is clear that the data surrounding the factors affecting the most efficient approach to generating a successful homologously recombining targeting vector are not clear and that disparities arise particularly between the data obtained on gene-targeting *per se* and data on intra- and extra-chromosomal recombination studies in tissue culture.

The lack of successful targeting of the Cyp2e1 gene by the $pHRPNS_{TK}$ Cyp2e1 construct could be attributed to a variety of potential shortfalls in the original vector's construction, or problems associated with the Cyp2e1 gene itself; if the problem lies in the targeting construct then this can be more easily remedied.

It is clear that the use of isogenic ES genomic DNA has a marked effect on the frequency of targeting; screening and recloning the Cyp2e1 from a genomic library prepared from isogenic DNA would therefore be the first step in preparing a new Cyp2e1 targeting construct.

Although the rationale behind the use of the 3' portion of the gene, based on the presence of the haem binding region of the Cyp2e1 protein appears logical, it is now clear that in a new construct the increase in the size of the Cyp2e1 gene employed in the targeting construct to around 14 kilobase-pairs, the seeming saturation point of the ES cells recombination system, may increase the targeting efficiency. It is of note however that successful targeting has been reported with homologous DNA fragments smaller than those used in the original $pHRPNS_{TK}$ Cyp2e1 construct (Hasty *et al.*, 1991). This size extension however, given that the coding region of the Cyp2e1 gene is approximately 11 kilobase-pairs in length, would have to be extended 5' of the gene to avoid the inclusion of the β2 SINE element in the 3' UTR of the gene. Such an increase in Cyp2e1 insert size would also necessitate the characterisation and sequencing of the entire Cyp2e1 gene both to identify restriction endonuclease sites, and to ascertain that no more SINE or other repetitive DNA elements were present.

In order to circumvent the selection of integrated non-functional mutated HSVTK genes, the inclusion of more than one copy of the gene may be necessary in a potentially more successful Cyp2e1 targeting construct. Other methods of selection are now being developed which may alleviate the problem of the selection procedure also selecting mutated vectors; one such approach is to use genes within the constructs which encode markers detectable by fluoresence activated cell sorting (FACS). This selection approach also removes the need to use markers conferring the ability to grow on medium which encourages the selection and clonal expansion of inactivating mutations within negative growth markers. A FACS selection approach also is more immediate, rather than requiring clonal expansion of surviving cells over a period of several days and the cells can be sorted as soon as a suitable time period to allow

marker expression has elapsed; to date however this approach has not yet been successfully applied in ES cells (Jasin *et al.*, 1990).

As the Cyp2e1 gene appears not to be expressed in the mouse embryo and the human and rat CYP2E1 gene is seen to be transcriptionally inactive embryonically, the chromatin structure around the gene may be highly compacted and structured making it unavailable to the recombinational machinery of the cell. If the problem in the lack of successful targeting of the Cyp2e1 gene does lie in the nature Cyp2e1 gene itself, no amount of targeting vector modification may surmount this obstacle.

Section 5.10: Summary

The results from the analysis of the Cyp2e1 subfamily in mouse illustrate the high level of conservation between the Cyp2e1 gene and predicted amino acid sequence and the orthologous CYP2E subfamily members from a variety of species. This high degree of conservation suggests that the CYP2E subfamily may perform similar functions and may potentially be regulated in a similar manner at a molecular level in a variety of species. These observations could be argued to validate the use of experimental animal systems to characterise both the potential behaviour, and the regulation, of the human CYP2E1 gene.

Although clearly unsuccessful in their stated objectives the two approaches adopted to enable the true role of the CYP2E subfamily in the initiation of carcinogenesis following exposure to CYP2E subfamily substrates are informative for future studies. Although it has been suggested that a stochastic balance must be struck to generate efficient holoenzyme generation of Cyp2e1 protein in bacterial systems the fact that Cyp2e1 holoenzyme is produced in *S. cerevisiae* can be further exploited. By increasing the levels of Cyp2e1 produced in this system a role for the CYP2E subfamily in the metabolism and activation of a series of environmental carcinogens could be established.

By improving the targeting system for the Cyp2e1 gene using some, or all of the suggestions made, it is possible that the Cyp2e1 gene could be removed from the mouse genome. Such an event would allow the establishment of a Cyp2e1 null-mouse line and the role of the CYP2E subfamily could be established in abstraction. This approach to study the role of the xenobiotic metabolising P-450 is potentially extremely powerful and will almost certainly be exploited successfully in the future.

Chapter 6: Summary and Future work

Section 6.1: Characterisation of the mouse Cyp2e1 subfamily and the *in vivo* regulation of the CYP2E subfamily, and other components of the xenobiotic metabolising system.

The molecular characterisation of the mouse Cyp2e1 cDNA and gene clearly demonstrated the high level of conservation present in the CYP2E subfamily; the level of conservation suggests that the Cyp2e1 enzyme may be expected to catalyse similar reactions, and be controlled in a similar manner to those CYP2E enzymes characterised in other species. The fact that the Cyp2e1 gene, in common with the CYP2E genes in most other species studied, is present in a single copy is significant. Other subfamilies of the P-450 family 2 have undergone an expansion in gene numbers suggested to have been driven by their role in the metabolism of xenobiotics in the diet (Gonzalez & Nebert, 1990; Matsunaga *et al.*, 1989). The high degree of conservation and the maintenance of a single gene in the CYP2E subfamily may reflect a more conserved, possibly endogenous, role for this subfamily.

Studies on the regulation of Cyp2e1 in the mouse demonstrated clearly that the CYP2E subfamily is subject to controls distinct from those observed previously to be operating on other xenobiotic metabolising P-450 families. Cyp2e1 protein levels were seen to be unchanged by chemicals such as phenobarbital, dexamethasone and 3-methylcholanthrene, which lead to elevations in the levels of other P-450. Cyp2e1 protein, but not mRNA, levels were seen to be elevated by treatment with acetone; the variety of the tissues in which Cyp2e1 protein was seen to be present and induced in, coupled with the potentially damaging nature of the products of CYP2E subfamily-catalysed reactions, has a clear implication for the ramifications of the role of the CYP2E subfamily in carcinogenesis. In starvation it was seen that both Cyp2e1 mRNA and protein levels were elevated; in both starvation and chemical induction it is thought that pre-existing CYP2E subfamily proteins are stabilised (Song *et al.*, 1989). The endogenous agent for this action may be acetone, which is a substrate for the CYP2E subfamily, and is produced as a result of elevated β-oxidation in starvation.

Future studies on the control of the Cyp2e1 subfamily in the mouse could analyse the effect of the diabetic state. Although it is possible to chemically induce diabetes in the mouse, strains have been identified which, in a similar manner to the BB rat, spontaneously become diabetic, such as the non-obese diabetic (NOD) mouse (Makino *et al.*, 1980; Wicker *et al.* 1987).

In studies on the effect of diabetes on a variety of xenobiotic metabolising enzymes in the BB/E rat, it was demonstrated clearly that endogenous signals produce profound effects on the activity of the xenobiotic metabolising system. The marked changes in the levels of these enzymes seen in diabetes would not be expected if the induced P-450 are viewed solely in terms of their ability to catalyse the metabolism of xenobiotics. However, by establishing possible roles for the P-450 enzymes as components of either intermediary metabolism, or metabolism controlling signalling pathways, the modifications in their levels observed in these situations can be rationalised.

In the observed elevations of the CYP2E and CYP4A subfamily levels it is possible that the enzymes perform endogenous roles relating to the elevation of fatty acid β-oxidation. In both these subfamilies it is possible to appreciate how an elevation in the enzymatic activity in the starved or diabetic state may allow the more efficient use of fuels by the body. The elevated CYP2E and CYP4A subfamily activities would allow increased acetone derived gluconeogenesis, and the generation of gluconeogenic products from β-oxidation respectively. Elevations in the other P-450 families seen could also perform a similar, but as yet undefined, role leading to increased efficiency in fatty acid metabolism. In this context it is of interest that the P-450 enzymes of many present day unicellular organisms are involved in intermediary lipid metabolism, and a similar role for the ancestral P-450 gene, from which the present day xenobiotic metabolising P-450 may have evolved, has been proposed (Gonzalez & Nebert, 1990).

An alternative role for the rationale behind the elevations seen may relate to an involvement of the P-450 enzymes in autocrine signalling. It is possible that the P-450 activities contribute to the generation of a co-ordinated switch in cellular metabolism in the starved or diabetic state from glucose towards fatty acids as a fuel source. The established involvement of P-450 in arachidonic acid metabolism (Fitzpatrick & Murphy, 1989; Nebert, 1990) and the presence of the the CYP4A subfamily in the pancreas, makes this an intriguing possibility.

Although the mechanisms for the transcriptional induction of the CYP1A genes (Hoffman *et al.*, 1991) and the CYP4A genes (Muerkoff *et al.*, 1992) are becoming more clearly understood and appear to involve inducible transcription factors from the steroid hormone superfamily, the molecular basis for transcriptional activation of, for example, some members of the CYP2B subfamily is still unclear. In the observed transcriptional induction of both the CYP4A and CYP2B subfamilies the affinity of the transcription factors for the inducing agents is very low; the inducing agents have been suggested to mediate their actions indirectly via other signalling systems (Okey, 1990; Gottlicher *et al.*, 1992). The observed induction of a variety of P450 families in diabetes, in which fatty acid levels are elevated, coupled with the very large number of 'orphan' steroid hormone receptors within a cell (O' Malley, 1990), suggest that the

molecular basis for the changes seen in several of the P-450 subfamilies may be the result of as yet uncharacterised internal-signalling, "paracrine", systems. The paracrine systems may use fatty acid molecules themselves, or metabolites, as the biologically active ligands. In this manner it is possible that the P-450 enzymes induced in diabetes may be acting as sensors for changes in ambient fatty acid levels within the body. When such changes are detected, the elevation of these enzymes and the increased generation of P-450 derived fatty acid metabolites, may act as signals to create a concerted shift in intermediary metabolism towards the use of fatty acids.

Future studies of the control of the CYP2E subfamily, and other components of the xenobiotic metabolising system could further expand these observations and suggestions. The proposed involvement of enzymes, such as the aldehyde and alcohol dehydrogenases, in the propandiol gluconeogenic pathway (Koop & Cassaza, 1986) make these components ideal candidates to extend the study of changes produced in starvation and diabetes on xenobiotic metabolising enzymes. Such analysis may allow the drawing together of enzymes of the xenobiotic metabolising system into an adaptive response to endogenously sensed metabolic changes; this response may form the basis for the induction of the xenobiotic metabolising enzymes resulting from the exposure to certain lipophillic xenobiotics.

The roles of the P-450 enzymes in paracrine signalling pathways could be investigated in isolated β-cell preparations. Using the release of insulin as an assay, the effect which general P-450 inhibitors, such as metyrapone, or isoform specific inhibitory antibodies produced on the response of the cells to metabolic stimuli could be monitored.

An appreciation of the endogenous controls operating on the xenobiotic metabolising system, whether they are vestigial or still metabolically pertinent, will allow a clearer understanding of situations in which the injurious actions of this enzyme system will become elevated.

Section 6.2: The molecular basis for the regulation of the CYP2E subfamily

The mechanism of substrate-induced CYP2E subfamily protein stabilisation

Chemicals which stabilise the CYP2E subfamily protein may potentially operate through their ability to mimic the actions of acetone; acetone, produced as a result of elevated fatty acid β–oxidation, may represent the endogenous substrate and controller of the CYP2E subfamily protein stability (Song *et al.*, 1989). Other than the observation that CYP2E subfamily proteins are seen to be stabilised by a variety of solvents, the molecular basis for this observation is unclear.

It was suggested that the CYP2E subfamily proteins were subject to rapid proteolysis as a result of phosphorylation of a serine residue. The actions of substrates and hormones, such as insulin, were suggested to reduce the level of phosphorylation at this site and so increase the stability of the CYP2E subfamily protein (Ingelman-Sunberg *et al.*, 1992). Experiments on mutant Cyp2e1 proteins lacking this proposed controlling phosphorylation site did not however substantiate this postulate. Both mutant and parental Cyp2e1 proteins were seen to accumulate to similar levels and to be lost at the same time-point in the expression system used. These studies also indicated that the suggested stabilising action of insulin on the CYP2E subfamily proteins may just reflect an overall increase, or decrease respectively, in the levels of protein translation and degradation within the cell.

The mutant Cyp2e1 protein expression system could be further characterised by a reduction in the time that a "pulse" of Cyp2e1 protein is produced. This could be achieved through the use of an inducible transient expression system and engineering instability motifs into the 3' UTR of the Cyp2e1 mRNA.

The fact that P-450 proteins can become phosphorylated has been known for many years; however to date this observation still appears to represent a phenomenon waiting for a defined functional role, if indeed such a role exists. Although tantalising, attempting to postulate a role for phosphorylation in the differential control of P-450 proteins suffers from an inherent lack of specificity. Nearly all family 2 P-450 proteins contain the proposed "controlling" phosphorylation site, and it is difficult to appreciate situations in which the cell could use such a post-translational change to modulate the activity of an individual P-450 subfamily.

The potential role of regulated message stability and translation in the CYP2E subfamily

The elevation of CYP2E mRNA levels in starvation and diabetes has been suggested to be the result of increased stability of the pre-existing CYP2E subfamily message (Song *et al.*, 1987). Two observations from this study suggest that this may not however represent the mechanism by which the CYP2E subfamily transcripts are induced in these situations:

i) studies in the BB/E rat demonstrated clearly that, in diabetes, the level of β-actin transcripts were elevated. The β-actin gene had been used as a presumed invariant transcription marker in the study to suggest that the CYP2E gene transcription level was unchanged (Song *et al.*, 1987).

ii) in the mouse it was seen that the Cyp2e1 transcript 3' UTR was interrupted by the insertion of a β2 repeat sequence; in all non-oligomeric protein encoding transcripts with regulated stability previously characterised, the motif controlling this parameter

was seen to be located within the 3' UTR (Hod & Hansen, 1986; Owen & Kuhn, 1987; Levin *et al.*, 1987; Bridges & Cudowski, 1989; Petersen *et al.*, 1989). The observation that Cyp2e1 mRNA levels were elevated in starvation suggests either that a stabilisation event in the CYP2E subfamily is mediated as a result of a regulatory motif elsewhere in the message, or that the elevation was the result of a modification in another control parameter, possibly transcriptional.
To further corroborate these suggestions, future work must include transcription "run-on" assays from nuclei isolated from starved or chemically induced diabetic animals. These assays would compare the level of CYP2E subfamily gene transcription to that of a gene, such as the microsomal GST, which has been demonstrated to be unchanged in starvation or diabetes. The inclusion in these assays of a gene, such as phosphoenolpyruvate carboxykinase (PEPCK), whose transcriptional activity is known to be elevated in these states, would further substantiate the results from such analysis.

The observations that solvent-induction of the CYP2E subfamily led to a decrease in mRNA but an increase in protein levels in some instances, and that CYP2E transcripts were seen to shift to a higher M_r polysomal band following chemical treatment, led to the suggestion that the CYP2E subfamily may be regulated by the translational initiation rate of its transcript (Kim *et al.*, 1990; Kim & Novak, 1990).
Analysis of the predicted RNA structures present within the 5' UTR of the CYP2E subfamily transcripts revealed a conserved stem-loop and the presence of a conserved NFκβ recognition site sequence motif. It is possible that the rate of translation initiation of CYP2E subfamily messages may be regulated by the differential binding of factors at this site, as previously characterised in the control of ferritin mRNA translation (Hentz *et al.*, 1987; Rauoult *et al.*, 1989; Brown *et al.*, 1989). If this is the case then this type of regulatory mechanism has previously been unreported in the P-450 superfamily.
The role of this 5' UTR element could be assessed through the coupling of the CYP2E subfamily 5' UTR to the 5' region of a reporter gene, such as chloramphenicol acetyl tranferase or luciferase, and studying the effect which solvent exposure produces on the level of reporter protein activity. Alternatively the effect of known inducers of NFκβ binding, such as exposure to oxidative stress, could be investigated (Meyer *et al.*, 1993). In this context it is of note that CYP2E1 protein levels were elevated on exposure of rats to hyperbaric oxygen (Tindberg *et al.*, 1989), analysis of the effect which such treatment produced on the CYP2E1 message levels would also be of interest.

Transcriptional regulation of the CYP2E subfamily

The similarity observed between the regulation of the CYP2E subfamily and gluconeogenic enzymes, such as PEPCK, prompted the analysis of the possible repressive action of insulin in the control of the transcriptional activity of the Cyp2e1 gene. Although the HepG2 cell line supported basal transcription from the Cyp2e1 promoter, this activity was unchanged by insulin. It is still not clear, for a variety of technical reasons relating to the phenotype of the cells in which the assay was conducted, whether this represents the true situation in the control of the CYP2E subfamily *in vivo*.

Future analysis of the Cyp2e1 promoter could either be conducted in the rat H4IIE cell line, previously seen to generate a reduction in PEPCK promoter activity following insulin exposure, or in the the HepG2 line with the inclusion of constructs containing insulin responsive PEPCK elements to demonstrate transcriptional modifications were occurring in a control promoter element.

Other approaches could be adopted to demonstrate an involvement of metabolism controlling hormones in the transcriptional regulation of the CYP2E subfamily. Foetal starvation *in utero* has been demonstrated to induce gluconeogenesis which, like the CYP2E activity, is only normally present *post partum* (Girard, 1986; Girard *et al.*, 1987). Using this approach, indirect evidence supporting a role for metabolic hormonal control of the CYP2E subfamily could be obtained.

The molecular basis for the observed sexual dimorphism in Cyp2e1 protein levels in the male and female mouse kidney could be analysed using the promoter obtained. Through the use of portions of the Cyp2e1 promoter, in conjunction with nuclear extracts isolated from male and female mouse kidneys, the elements in the promoter contributing to the differential sexual control of this gene could be established using band shifting assays.

Section 6.3: Assessing the role of the CYP2E subfamily in chemical mutagenesis

The attempts to establish bacterial and yeast systems expressing the Cyp2e1 protein to allow the analysis of the role of the CYP2E subfamily in chemical mutagenesis were not successful in their stated objectives. In bacterial expression systems, a balance between production of excessive and insufficient protein levels was not achieved and detectable haem incorporation in the apoprotein generated could not be detected. Given the greater sensitivity of the bacterial system to chemical damage and the well characterised mutagenicity assays available, the expression of active Cyp2e1 in this system is desirable. Future analysis of this system could be undertaken empirically; by

employing a variety of different Cyp2e1 constructs eventually active Cyp2e1 protein may be generated. Alternatively it may be possible to exploit constructs previously seen to generate active mammalian P-450 in bacteria. Through the insertion of a portion of the Cyp2e1 cDNA into a vector containing the N-terminal region of a P-450 which had been successfully expressed in bacteria it is possible that an active Cyp2e1, retaining catalytic integrity, may be produced.
Detectable levels of haem were seen to be present in *S. cerevisiae* expressing Cyp2e1 protein. The levels of Cyp2e1 protein produced by this system could be elevated, either by the modification of the context of the initiation methionine of the message, or by inserting a portion of the Cyp2e1 cDNA into a vector containing the sequence encoding the N-terminal membrane anchor of the endogenous yeast CYP51 P-450. Following the generation of a unicellular tester strain with demonstrable Cyp2e1 activity towards model substrates, such as *p*-nitrophenol, a clear idea of the role of the Cyp2e1 protein in the generation of chemical mutagenicity can be established.

By producing a mouse in which the Cyp2e1 gene had been removed by a homologous targeting event the role of the CYP2E subfamily in carcinogenesis, by abstraction, could elegantly be demonstrated. The characterisation of this experimental approach by this study has highlighted the requirements which, assuming that the Cyp2e1 gene is not refractory to a recombination event, future experiments may need to adopt.
The future Cyp2e1 targeting vector will require the cloning and complete characterisation of the Cyp2e1 gene from a genomic library constructed from C129 mice so that the construct contains DNA fragments isogenic to the C129 derived embryonic stem cells. Through the inclusion of the whole of the Cyp2e1 gene, around 11,000 base-pairs, in the construct, the possibility of a successful targeting event will be maximised as the size requirements of the recombinational machinery would be more than adequately met. In addition by inserting several copies of the negative selection gene into the construct, the selection of randomly integrated constructs with mutated negative selection markers may also be diminished.
The generation of null-phenotype mouse strains in this manner, assuming that recombination is physically possible, will almost certainly prove successful in the future. These studies will allow the study of the loss of a particular P-450 isoform on carcinogenesis, which has been exploited greatly in epidemiological studies in the human (Wolf, 1989; Smith *et al.*, 1992), to be applied in experimental animals. Through the use of this approach the role of particular P-450 isoforms in the generation of chemical carcinogenesis can move from reductionist approaches involving unicellular organisms, with all the problems inherent in these systems, into the whole mammal.

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Appendix: Amino acid data and abbreviations used in this work

name	code	side group	MW	KD
alanine	A ala	-CH3	71.0	1.8
cysteine	C cys	-CH2-SH	103.0	2.5
aspartic acid	D asp	-CH2-COOH	115.0	-3.5
glutamic acid	E glu	-CH2-CH2-COOH	129.0	-3.5
phenylalanine	F phe	-CH2-phi	147.1	2.8
glycine	G gly	-H	57.0	-0.4
histidine	H his	-CH2-imidazole	137.1	-3.2
isoleucine	I ile	-CH(CH3)-CH2-CH3	113.1	4.5
lysine	K lys	-(CH2)4-NH2	128.1	-3.9
leucine	L leu	-CH2-CH(CH3)2	113.1	3.8
methionine	M met	-CH2-CH2-S-CH3	131.0	1.9
asparagine	N asn	-CH2-CONH2	114.0	-3.5
proline	P pro	[N]-(CH2)3-[CH]	97.1	-1.6
glutamine	Q gln	-CH2-CH2-CONH2	128.1	-3.5
arginine	R arg	-(CH2)3-NH-CNH-NH2	156.1	-4.5
serine	S ser	-CH2-OH	87.0	-0.8
threonine	T thr	-CH(CH3)-OH	101.0	-0.7
valine	V val	-CH-(CH3)2	99.1	4.2
tryptophan	W trp	-CH2-indole	186.1	-0.9
unknown	X ---		0.0	0.0
tyrosine	Y tyr	-CH2-phi-OH	163.1	-1.3

MW: Molecular weight (Da).
KD: Kyte and Doolittle assigned hydrophobicity index.

DEDUCED AMINO-ACID SEQUENCE, PREDICTED STRUCTURAL FEATURES AND PROPOSED SUBSTRATE BINDING RESIDUES OF MOUSE Cyp2e1

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Summary: The deduced amino sequence of mouse Cyp2e1 was obtained and areas within the protein which may contribute to the binding of the haem and substrates were determined by comparison of aligned CYP2E1 sequences with the crystallographically solved P450cam structure.

Introduction: Certain Cytochrome P-450 (P450) appear to be part of an adaptive response to chemical challenge and exposure to a foreign compound may thus induce P450 species active in its metabolism. Members of the CYP2E1/Cyp2e1 subfamily were shown to be induced by and metabolise a variety of organic compounds for example acetone, chloroform and ethanol. This subfamily was also shown to metabolise and activate several carcinogenic nitrosamines, for example *N* - nitrosodimethylamine (NDMA), and hepatotoxins such as acetominophen, carbon tetrachloride and benzene (Reviewed in [1]). To begin to study the role of this protein in xenobiotic metabolism in the mouse and relate this to the role played in other species the cDNA encoding the Cyp2e1 protein was cloned. Alignment of the deduced Cyp2e1 amino acid sequence and the CYP2E1 sequences of other species in conjunction with the crystallographically solved structure of P450cam, (CYP101, [2]), allowed the prediction of regions within Cyp2e1/CYP2E1 potentially involved in the haem environment and the binding of substrates.

Materials and Methods: Partial *Cyp2e1* cDNA clones were obtained from a C57BL/6 male liver library in λgt11 using the full-length human *CYP2E1* as a probe [3]. Using the partial clones as probes a full-length *Cyp2e1* clone was obtained from a BALB/c male liver library in λZAP II. Single-stranded DNA was prepared from the clone and sequenced in both directions using the Sanger dideoxy-termination method with a series of synthetic oligonucleotide primers. Sequences were compiled and analysed using the GCG [4] and GeneJockey (Biosoft) software packages.

Results and Discussion: The deduced mouse Cyp2e1 amino acid sequence is illustrated in Figure 1. The enzyme contains 493 amino-acids with a predicted molecular mass of 56, 781 Daltons. The mouse sequence displayed 92, 77, 79 and 79% identity to the rat [3], human [3], rabbit gene 1 and gene 2 [5] CYP2E1 protein sequences respectively. The sequences of these CYP2E1 protein sequences, rabbit CYP2C5 (a Progesterone 21-hydroxylase, [6]), and P450cam were aligned according to the method discussed in Zvelebil *et al.* [7]. This alignment allowed the prediction of α -helical regions of Cyp2e1/CYP2E1 by analogy to the crystallographically determined structure of P450cam, shown horizontally boxed in Figure 1 using the nomenclature of Poulos *et al.* [2].

1) The predicted haem environment of Cyp2e1/CYP2E1: In P450cam the haem is sandwiched between the L and I helices with the GGLDT (sequence around 244c, "c" donating P450cam amino-acid number, in Figure 1.) forming the principal contacts. The corresponding haem contacts within the

Figure 1: Mouse Cyp2e1, and rat, human, rabbit gene 1 and gene 2 CYP2E1, rabbit CYP2C5, and P450cam sequences were aligned. Differences between the mouse Cyp2e1 and the CYP2E1 of other species are shown and a consensus generated. P450cam structural elements are horizontally boxed and labelled according to Poulos *et al.* [2]. Predicted haem and substrate binding sites are vertically boxed. The canonical cAMP dependant kinase phosphorylation site is indicated ****. Family 2 amino-acids are labelled to the left and right; numbered P450cam amino acids contain a "c" post-script.

predicted I helices of the Cyp2e1/CYP2E1 are AGTET. The haem propionate groups in P450cam are surrounded by 5 hydrogen bond donors: R112c, R299c, H355c, Q108c, and D297c. Three of these five bacterial residues contain aligned mammalian residues that could perform a similar function in Cyp2e1/CYP2E1: R112c/R135, R299c/H369, and H355c/R434. Similar bonds could not be provided by the aligned W131/Q108c and L367/D297c. The residues potentially contributing to the haem environment are vertically boxed in Figure 1. It is interesting that a canonical cAMP dependent protein kinase site (Indicated by *** and S129* in Figure 1.) is near to one of the analagous haem propionate bonding R112c/R135 and the Q108c/W131 site, active as a bond donor in P450cam but not the mammalian protein. The possibility that a potential phosphorylation event at S129 may affect haem/P450 reductase interaction is intriguing but remains to be elucidated.

2) Amino-acids contributing to a putative Cyp2e1/CYP2E1 substrate binding site: Residues involved in binding the P450cam substrate, camphor, were identified crystallographically [2]. Residues making major contacts with the camphor carbonyl oxygen atom and their corresponding mammalian counterparts are: F87c/F106 in Cyp2e1/CYP2E1 but L106 in rabbit CYP2C5 and CYP2E1 gene 2, Y96c/I114 and L244c/D295 in all the mammalian P450. Other contacts in the bacterial crystal occurred at: T185c/R233 in Cyp2e/CYP2E but K233 in rabbit CYP2C5, V295c/S366 in Cyp2e1/CYP2E1 but T366 in rabbit CYP2C5, V247c/F298 in Cyp2e1/CYP2E1 but G298 in rabbit CYP2C5 and D297/L368 in all the mammalian P450 aligned. In all but one case in the Cyp2e1/CYP2E1 family, F87c/F106 in all Cyp2e1/CYP2E1 but L106 in rabbit gene 2, the putative substrate binding residues are invariant. Cyp2e1/CYP2E1 substrates have been shown to vary between species, for example rat CYP2E1 metabolises pyrazole whilst mouse Cyp2e1 does not [8]. The fact that different species CYP2E1/Cyp2e1 metabolise different substrates yet putative substrate binding residues remain invariant suggests that other as yet unassigned amino-acids may also play a role in determining the enzyme's substrate specificity.

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